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(54) Title: IMMOBILIZED SILVER IMMUNOASSAY SYSTEM

(57) Abstract

The present invention provides bioassay plates having silver ions immobilized thereon, which are useful in immunoassays for detection of antibodies or antigens. Methods of making and using the biossay plates are also provided, as well as an apparatus for making activated bioassay plates.

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IMMOBILIZED SILVER IMMUNOASSAY SYSTEM

Specification

Background of the Invention

Since the late 1950s, when the work of Rosalyn Yalow and Solomon Berson (Yalow et al., 1959, Nature (London) 184:1648; Yalow et al., 1960, J. Clin. Invest 39:1157) first illuminated the possibilities of immunoassays, immunodiagnostic techniques based on the specific interaction of antibody and antigen have become of paramount importance in the clinical, agricultural, food, veterinary, and environmental sectors. In 1996, it was estimated that the worldwide market of immunoassay products was $10 billion in the clinical sector alone, and increasing at an annual rate of 10% (Deshpande, Enzyme Immunoassay: From Concept to Product Development, New York:Chapman & Hall, 1996). This market is driven by an ever-increasing desire for assays of greater sensitivity and specificity, at reasonable financial costs. It is in this environment that the specific and irreversible interaction between avidin or streptavidin and biotin has found use.

Streptavidin, a close relative of egg white avidin, is expressed in *Streptomyces avidinii* (Green, 1990, Methods in Enzymology 184:51), and both avidin and streptavidin exhibit an affinity for biotin on the order of 10^{15} M^{-1}. The streptavidin-biotin system has become a widely-used tool of molecular biology in such applications as affinity chromatography, cytometry, nucleic acid research, and diagnostics (Diamandis et al., 1991, Clin. Chem. 37:625; Wilchek et al., 1988, Anal. Bio Chem. 171:1). A common immunological procedure calls for the use of streptavidin-coated microtiter plates, which are used to capture either biotinylated antibodies or antigens. Since the assay is based on the interaction of streptavidin and biotin, universal kit-based assay formats are possible. These universal assays are also the basis of many automated immunological testing systems (Chan, ed., 1996, Immunoassay Automation: An Updated Guide to Systems, San Diego: Academic Press, 51-308). A format utilizing a microtiter-based enzyme-linked immunosorbent

assay (ELISA) can measure a wide variety of analytes using a dual antibody or "sandwich" immunoassay. The choice of a streptavidin-coated solid support is made to overcome the limitations present in the direct antibody coating of polystyrene supports, which can result in unreliable or nonuniform coating of the solid support, in addition to the steric affects of binding upon the antibody. However, while streptavidin systems allow for universal ELISA kits and can improve assay sensitivity, coated plates can be costly.

Research examining the behavior of biotin has found that immobilized silver ions will bind biotinylated compounds both strongly and, in this case, reversibly. This research has been done with both immobilized metal affinity chromatography (IMAC) (Garcia et al., 1994, Reactive Polymers 23:249; Kim et al., 1995, Art. Cells, Blood Subs., and Immob. Biotech. 23:555; Miles et al., 1995, J. Chromatogr. A. 702:173) and paramagnetic particles (Ramirez-Vick, 1997, Ph.D. Dissertation, Arizona State University, Tempe, AZ). It has been discovered in accordance with the present invention that immobilized silver ions can be used in an immunoassay format to provide a sensitive and inexpensive universal assay.

Summary of the Invention

The present invention provides an immunoassay system comprising bioassay plates having silver immobilized thereon. The present invention further provides a method of making bioassay plates having silver immobilized thereon.

In another embodiment, the present invention provides a method for detecting an antigen or antibody, and a kit useful for the detection of an antigen or antibody.

An apparatus for providing activated bioassay plates is also provided by the present invention.

Brief Description of the Drawings

Fig. 1 is a graph depicting the sensitivity of a microtiter plate having silver immobilized thereon.

Fig. 2 is a schematic of an immunoassay utilizing microtiter plates having silver immobilized thereon, and biotinylated capture antibodies.

Fig. 3 is a graph providing a kinetic analysis of a horseradish peroxidase immunoassay.

Fig. 4 is a schematic of a checkerboard assay.

Fig. 5 shows the results of a checkerboard assay.

Fig. 6 is a schematic depicting the arrangement of activated and control wells of a microtiter plate.

Fig. 7 is a graph of immunoassay results comparing a streptavidin method with the silver method of the present invention.

Fig. 8 is a flow diagram describing the apparatus of the present invention.

Fig. 9 is a side view of the apparatus of the invention.

Fig. 10 is a diagram of the liquid handling system of the invention.

Figs. 11a-11c are diagrams of the liquid transfer manifold of the invention. Fig. 11a is a side view of the reagent addition stage and vacuum stage; Fig. 11b is a front view of the reagent addition stage; Fig. 11c is a front view of the vacuum stage.

Detailed Description of the Invention

The present invention provides an immunoassay system comprising bioassay plates having silver, in particular silver ions, immobilized thereon. The invention further provides methods of making and using such bioassay plates. The bioassay plates and immunoassay of the present invention are useful for the detection of antibodies and antigens, and provide cost and sensitivity advantages relative to the streptavidin-coated bioassay plates of the prior art.

The bioassay plates used in the present invention are microwell, or microtiter, plates known in the art for immunoassays, and are commercially available. Conventional microwell plates are 96-well microplates having wells arranged on an 8 x 12 matrix on 9 mm centers. Each well holds approximately 300 microliters. 384-well plates are also available, in which the wells are arranged in a 16 x 24 matrix on a 4.5 mm center, with each wells having a brim volume of approximately 80 microliters. Well plates defined by larger matrices, e.g. 1536 well plates, are also available. The number and configuration of the wells are not critical to the present

invention, and are used by way of example only. The bioassay plates used in accordance with the present invention are plastic, and preferably polystyrene.

Bioassay plates having silver ions immobilized thereon are made by a method comprising functionalizing a multi-well bioassay plate to provide an amine-containing bioassay plate, adding polymerized glutaraldehyde to the wells of the plate for a time and under conditions whereby the amines are activated by glutaraldehyde, rinsing the plates with an aqueous solution, adding thiourea to the wells of the plate for a time and under conditions whereby the thiourea is reacted with a glutaraldehyde moiety of the glutaraldehyde-activated bioassay plate, rinsing the plate with an aqueous solution, and contacting the plate with silver ions for a time and under conditions whereby the silver ions are immobilized on said plate.

The bioassay plate may be constructed of any material that can be functionalized to contain an amine group. In a preferred embodiment, the multi-well bioassay plate is a plastic multi-well bioassay plate. For example, the plate may be made of polystyrene, polyethylene, polypropylene, or other primary polymers or composite resins. Polystyrene is particularly preferred. Methods for functionalizing these materials to contain an amine group are known in the art. For example, a polystyrene bioassay plate can be functionalized to contain an amine group by methods known in the art and disclosed for example in Immobilized Affinity Ligand Techniques, Hermanson et al., eds., San Diego: Academic Press, 1992, the disclosure of which is incorporated herein by reference. Aminated polystyrene bioassay plates are also commercially available, for example from Corning (Corning, NY), NUNC (Denmark) and Micro Membranes (Newark, NJ). Preferably the plate is amidated or aminated to contain from about 1×10^{13} to 1×10^{14} amine sites per cm^2.

Polymerized glutaraldehyde may be prepared by allowing glutaraldehyde (25 wt %) to polymerize, for example for from 1 to 36 hours at from 23°C to 70°C. In a preferred embodiment polymerization is at 70°C for about 24 hours. The polymerized glutaraldehyde is added to the wells of the plate and incubated under conditions whereby a glutaraldehyde-activated plate is produced, for example for from 1 to 36 hours at from 23°C to 70°C. In a preferred embodiment, incubation is at 35 to 50°C for 1 to 24 hours, and more preferably at about 37°C for about 24 hours. The

plate is rinsed with an aqueous solution, for example deionized water, to remove unreacted glutaraldehyde. The wells of the plate are then filled with thiourea, for example from 0.01M to 1M solution, and preferably a 1M solution, under conditions suitable for reaction with the glutaraldehyde moiety, for example for from 1 to 36 hours at from 23°C to 70°C. In a preferred embodiment at, incubation is for 1 to 24 hours at 35 to 50°C, and more preferably about 24 hours at about 37°C. The plate is rinsed with an aqueous solution, for example deionized water, to remove unreacted thiourea.

Silver ions, preferably in the form of silver nitrate, are then added to the plate under conditions whereby silver ions are immobilized on the plate, for example for from 1 to 36 hours at from 23°C to 70°C. In a preferred embodiment, incubation is for about 24 hours at about 37°C. The plates are then rinsed with an aqueous solution, for example deionized water, and may be stored until use, preferably in an opaque sleeve.

The bioassay plates having silver ions immobilized thereon are useful in a method for detecting an antigen or an antibody. It has been discovered in accordance with the present invention that the silver ions immobilized on bioassay plates are capable of strong binding to biotinylated antibodies and antigens. Accordingly, the plates of the invention may be used in standard enzyme-linked immunosorbent assays (ELISAs). For example, a bioassay plate having silver ions immobilized thereon is incubated with biotinylated antibody to provide a bioassay plate having the antibody immobilized thereon. After washing with an aqueous solution, the plate is incubated with a solution containing the cognate antigen under conditions whereby the antigen binds to the immobilized antibody, followed by another washing step. The antigen is then detected, for example by subsequent incubation with a labeled antibody having specificity for the antigen. Detectable labels for antibodies are known in the art and include radiolabels, fluorescent tags, and enzyme conjugates. In a preferred embodiment, the aqueous solution contains deionized water and Tween (polyoxyethylene sorbitan monolaurate).

An antibody may be detected using the plates of the present invention in an indirect ELISA assay. For example, a bioassay plate having silver ions immobilized

thereon is incubated with a biotinylated antigen to provide a bioassay plate having the antigen immobilized thereon. After washing with an aqueous solution, the plate is incubated with a solution containing the cognate primary antibody under conditions whereby said antibody binds to the immobilized antigen. After incubation and washing, a labeled secondary antibody is added and incubated under conditions whereby it binds to the primary antibody. After washing, the secondary antibody is detected, wherein detection thereof indicates the presence of the primary antibody. Detectable labels for antigens are known in the art and include radiolabels, fluorescent tags, and enzyme conjugates. In a preferred embodiment, the aqueous solution contains deionized water and Tween.

Conditions for biotinylating antibodies and antigens are well known in the art and disclosed, for example, by Bayer et al., "Protein Biotinylation" (1990) Methods in Enzymology 184:138 and O'Shannessy "Antibodies Biotinylated via Sugar Moieties" (1990) Methods in Enzymology 184:162, the disclosures of which are incorporated herein by reference. Conditions for performing ELISAs are well-known in the art and disclosed, for example, by Harlowe, et al., (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, the disclosure of which is incorporated herein by reference.

The present invention further provides a kit useful for the detection of an antigen or antibody. The kit comprises, in a first container, a bioassay plate having silver ions immobilized thereon. In a preferred embodiment, the bioassay plate is a polystyrene multi-well plate. The kit may optionally contain a second container containing a biotinylated antibody or a biotinylated antigen. The kit may optionally contain a third container containing labeled antibody, when the second container contains a biotinylated antibody, or a labeled secondary antibody, when the second container contains a biotinylated antigen.

The present invention further provides an apparatus useful for the automated production of microplates having modified surface chemistry. As shown by the flow diagram in Fig. 8, the apparatus provides for filling the wells of a microplate with a reagent in an addition/withdrawal chamber; conveying the microplate to an incubation chamber in which the microplate is sealed, heated and agitated, and unsealed;

conveying the microplate to the addition/withdrawal chamber for evacuation of reagent, washing, and addition of a second reagent; conveying the microplate to the incubation chamber for sealing, heating and agitation, and unsealing; conveying the microplate to the addition/withdrawal chamber for evacuation of reagent and washing; followed by subsequent cycles of reagent addition and incubation, or conveyance of the microplate out of the machine.

In a preferred embodiment, and with reference to Figs. 9, 10, 11a, 11b and 11c, the apparatus comprises a housing having disposed therein a reagent addition/withdrawal chamber (1) and an incubation chamber (2). Microplates are conveyed into and between the chambers by means of a plate holder (3) movable horizontally by a plate holder track (4). Reagents and wash solution are provided in storage containers (5) connected by reagent lines (13) to the dispense portion of a manifold (6) which delivers reagent and wash solution by dispense lines (7) by means of a liquid pump (11). After reagent addition, microplates positioned on the plate holder (3) are conveyed via the plate holder track (4) into the incubation chamber (2). The microplate is sealed by a non-reactive sealing plate (13) delivered vertically. The incubation chamber further provides a means for heating and agitating the microplate (14). After a time predetermined by the user, the microplate is conveyed to the reagent addition/withdrawal chamber (1) via plate holder track (4). Spent reagent is removed through aspirator lines (8) and withdrawn by the aspirate portion of the same manifold (6) by means of a vacuum pump (9) through waste lines (12) to a waste container (10). Wash solution is added through the dispense portion of the manifold (6) which delivers wash solution through dispense lines (7). Wash solution is removed through aspirator lines (8) and withdrawn by the aspirate portion of manifold (6) by means of a vacuum pump (9) through waste lines (12) to a waste container (10).

The foregoing steps are carried out in an automatic programmed manner under the control of electronic circuitry contained in the housing.

All references cited herein are incorporated in their entirety.

The following examples serve to further illustrate the present invention.

Example 1

A polystyrene 96 well microtiter plate aminated to provide approximately 2 x 10^{13} active amine sites per cm^2 was obtained from Corning (Corning, NY). Glutaraldehyde (25 wt %) which had been allowed to polymerize at 70°C for 24 hours was added to each well of the microplate, which was then incubated at 37°C for 24 hours to facilitate plate activation. The plate was then rinsed with deionized water and wells filled with a 1M solution of thiourea, followed by an additional 24 hour period of incubation at 37°C. After another rinsing of the plate, a 1M solution of silver nitrate was allowed to contact the plate during another 24 hour incubation at 37°C. The plate was then rinsed extensively.

The biotin-binding capability of such an immobilized silver microtiter plate was tested as follows. A complete plate was assembled from stripwells, using alternating strips of unactivated and silver-containing wells, where the silver-containing strips were in the odd-numbered rows and the unactivated strips were in the even-numbered rows. The test consisted of the binding of biotinylated horseradish peroxidase (bHRPO), which was detected using the chromogenic reaction of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS®). This was a one-step, unamplified assay; 150 µl of the bHRPO solution was added to the wells using doubling dilutions, and allowed to bind for one hour. Following this, the plates were rinsed with deionized water and the ABTS® in citrate buffer was added. The developed color in the wells was read after one hour at room temperature using a BioRad Benchmark Microplate Reader set at 415 nm. The plate setup and moles of bHRPO corresponding to each dilution are shown in Tables I and II, respectively.

TABLE I

	1	2	3	4	5	6	7	8	9	10	11	12
A	no bHRPO		no bHRPO		no bHRPO		no bHRPO		no bHRPO		no bHRPO	
B	no bHRPO		1 / 1		1 / 64		1 / 4096		1 / 262144		no bHRPO	
C	no bHRPO		1 / 2		1 / 128		1 / 8192		1 / 524288		no bHRPO	
D	no bHRPO		1 / 4		1 / 256		1 / 16384		1 / 1048576		no bHRPO	
E	no bHRPO/ABTS		1 / 8		1 / 512		1 / 32768		1 / 2097152		no bHRPO/ABTS	
F	no bHRPO/ABTS		1 / 16		1 / 1024		1 / 65536		1 / 4194304		no bHRPO/ABTS	
G	no bHRPO/ABTS		1 / 32		1 / 2048		1 / 131072		1 / 8388608		no bHRPO/ABTS	
H	no bHRPO/ABTS		no bHRPO/ABTS		no bHRPO/ABTS		no bHRPO/ABTS		no bHRPO/ABTS		no bHRPO/ABTS	

TABLE II

Dilution	Grams bHRPO	Moles bHRPO
1 / 1	2.40E-06	5.33E-11
1 / 2	1.20E-06	2.67E-11
1 / 4	6.00E-07	1.33E-11
1 / 8	3.00E-07	6.67E-12
1 / 16	1.50E-07	3.33E-12
1 / 32	7.50E-08	1.67E-12
1 / 64	3.75E-08	8.33E-13
1 / 128	1.88E-08	4.17E-13
1 / 256	9.38E-09	2.08E-13
1 / 512	4.69E-09	1.04E-13
1 / 1024	2.34E-09	5.21E-14
1 / 2048	1.17E-09	2.60E-14
1 / 4096	5.86E-10	1.30E-14
1 / 8192	2.93E-10	6.51E-15
1 / 16384	1.46E-10	3.26E-15

TABLE II

Dilution	Grams bHRPO	Moles bHRPO
1 / 32768	7.32E-11	1.63E-15
1 / 65536	3.66E-11	8.14E-16
1 / 1E+05	1.83E-11	4.07E-16
1 / 3E+05	9.16E-12	2.03E-16
1 / 5E+05	4.58E-12	1.02E-16
1 / 1E+06	2.29E-12	5.09E-17
1 / 2E+06	1.14E-12	2.54E-17
1 / 4E+06	5.72E-12	1.27E-17
1 / 8E+06	2.85E-13	6.36E-18

After applying statistical curve fitting techniques, a concentration activity curve was generated and is shown in Figure 1. Results are presented in terms of moles of bHRPO. As demonstrated therein, the detection limit of this system approaches femtomolar levels, even using an unamplified system. The typical Gaussian response curve of ELISA is also seen in Figure 1, which further demonstrates that the maximum sensitivity of this assay occurs at bHRPO levels of 2 x 10^{-13} moles.

Example II

Example I demonstrated that immobilized silver microtiter plates are capable of binding biotin and a biotinylated antigen. This example demonstrates that biotinylated capture antibodies can be bound to immobilized silver microtiter plates and used for antigen capture.

Immobilized silver microtiter plates were prepared as described in Example I. The plates were incubated with biotinylated anti-peroxidase antibodies (Jackson Immunological) at an antibody concentration of 1.2 mg/ml diluted 1:100, with addition of 150 microliters to each well for an hour. After washing the plates with 50 mM phosphate buffer with 0.1% v/v Tween 20 detergent (Phosphate/Tween buffer), the enzyme horseradish peroxidase (Sigma) was added, in the amounts shown in

Table III, for one hour incubation. Phosphate/Tween buffer was used as the dilution buffer in the assay.

The results of eight assays of the type shown in Figure 2 are presented in Figure 3, along with 2σ error limits. The data for the immunoassays is shown in Table III, using the eight simultaneous assay replications for a basic statistical analysis. Figure 3 uses kinetic rates to determine the detection of enzyme, which allows for a linear fit of the data when presented on a semi-log plot using the dilution number of the initial enzyme solution. At the initial dilutions, the margin of error is rather high, due to the high amounts of enzyme in the initial solution (2.56×10^{-11} moles per well) producing extremely rapid kinetics. As the enzyme goes through doubling dilutions, the kinetics are measured more easily and the precision of the assay improves at dilution number 16 (1.6×10^{-12} moles per well). The detection limit of this assay is in the region of dilution number 1000 (0.25 femtomoles), which approaches the theoretical detection limit of the enzyme substrate system being used. (Deshpande, Enzyme Immunoassays: From Concept to Product Development, New York, Chapman & Hall (1996), 1-422.

TABLE III

Kinetic Immunoassay Data

Dilution Number	HRPO (g/well)	HRPO (mol/well)	Kinetic Rate	2σ Limits
1	1.13E-06	2.56E-11	927.65	286.73
2	5.63E-07	1.28E-11	773.66	253.20
4	2.81E-07	6.40E-12	666.47	95.75
8	1.41E-07	3.20E-12	598.88	97.22
16	7.03E-08	1.60E-12	551.07	61.58
32	3.52E-08	8.00E-13	452.40	25.79
64	1.76E-08	4.00E-13	361.72	24.07
128	8.79E-09	2.00E-13	277.00	20.58
256	4.39E-09	1.00E-13	193.03	27.47

TABLE III

Kinetic Immunoassay Data

Dilution Number	HRPO (g/well)	HRPO (mol/well)	Kinetic Rate	2σ Limits
512	2.20E-09	5.00E-14	128.36	49.72
1024	1.10E-09	2.50E-14	80.85	53.07
2048	5.49E-10	1.25E-14	65.28	62.02
4096	2.75E-10	6.25E-15	60.26	61.08
8192	1.37E-10	3.12E-15	62.54	60.02
16384	6.87E-11	1.56E-15	52.25	78.54
32768	3.43E-11	7.81E-16	46.92	83.12
65536	1.72E-11	3.91E-16	36.93	69.52
131072	8.58E-12	1.95E-16	40.52	69.90

The basis of the foregoing is the assumption that the enzyme binds only to the antibodies, which specifically select it from the test solution. In order for this to be valid, background binding of the enzyme to the plate must be at insignificant levels. In order to determine this, a checkerboard assay was performed (Deshpande, supra). The assay arrangement is shown in Figure 4. When performed, the initial concentrations of the antibody and the enzyme were the same as described above, as were all buffers and incubation periods. The checkerboard assay, which is primarily visual, is a means to determine the effect of the antibody concentration and the enzyme concentration on the assay results. As seen in Figure 5, the enzyme only binds when antibody is present in the well. As antibody concentrations decrease horizontally across the plate, enzyme binding falls rapidly, as shown by the absence of color on the right side of the plate, even at the extreme enzyme concentrations introduced into row A.

Example III

The foregoing examples demonstrate that immobilized silver is capable of binding biotin in the bHRPO assay and that effective immobilization of capture antibodies in the immobilized silver microplate wells is possible. In the present example, the silver ion immunoassay format is then compared to the current streptavidin technology used to bind biotinylated antibodies. Plates coated with streptavidin (Xenopore, XPS 010 00) were obtained for this purpose. These plates represent the best of the currently available products since they feature a covalent linkage between the streptavidin and the plate surface, and are also blocked with a proprietary nonbiotinylated protein to inhibit background binding. No blocking agents are used on the silver plates. A side by side comparison with an immobilized silver plate prepared according to Example I was performed as follows.

Both the streptavidin plate and the silver plate were hydrated and rinsed with 50 mM pH 7 phosphate buffer. The wells in the plates were filled to capacity with buffer and allowed to stand at room temperature for 10 minutes. The plates were then rinsed twice with the same buffer.

Biotinylated anti-peroxidase antibodies (Jackson Immunological) were used to coat the plates. In this assay, 150 µl of antibody solution (1.2 mg/ml) diluted to 1:100 was added to the wells in the odd numbered columns. The even numbered columns were used as control wells, and were filled with 150 µl of buffer. This arrangement of activated and control wells is shown in Figure 6. The buffer used to dilute the antibodies and fill the control wells was 50 mM pH 7 phosphate buffer with 0.1% v/v Tween 20 Phosphate/Tween) added to inhibit any hydrophobic binding in the plates. The plates were then covered and allowed to stand at room temperature for 1.5 hours.

Both plates were thoroughly washed using a Bio-Rad Plate Washer filled with Phosphate/Tween buffer as the wash buffer. Horseradish peroxidase (Sigma) was used as the antigen in this test. A solution of peroxidase was created by adding 8 x 10^{-4}g of the enzyme to 10 ml of Phosphate/Tween. 1 ml of this solution was then added to 9 ml of buffer. 300 µl of the diluted enzyme solution was added to wells A1, A2, A5, A6, A9, and A10. The amount of enzyme added to these primary wells is shown in Table IV. The remainder of the wells were filled with 150 µl of

Phosphate/Tween. For Test 1, 150 µl of solution was withdrawn from A1 and A2 and diluted into B1 and B2. This procedure was repeated down the plate, with the solution from H1 and H2 carried to wells A3 and A4. Tests 2 and 3 were performed in a similar fashion. After all of these doubling dilutions were complete, the plates were covered and allowed to stand at room temperature for 1 hour.

For enzyme detection the plates were first washed using the automated plate washer with Phosphate/Tween. Then, 150 µl of an ABTS solution was added to each well in the plate, and the rate of formation of the colored product read at 415 nm in a Bio-Rad Benchmark Microplate Reader. Readings took place every 15 seconds for 5 minutes. The ABTS solution was made by adding 17 mg of ABTS to 100 ml of 50 mM pH 5 citrate buffer. Immediately before use, 100 µl of 3% hydrogen peroxide was added to the solution to catalyze the enzymatic reaction.

The raw data for these experiments is presented in Table IV and Table V. Using the average of the normalized tests, a plot comparing the streptavidin plate to the silver plate was prepared. The data points shown on the graph in Figs. 7 are the average values of the normalized tests shown in Tables IV and V. The exception to this are the data points taken at Dilution 1, which were replaced by the values representing the asymptotic approach of the immunoassays as determined from the preceding points. From this, the maximum kinetic rate achievable in the silver plate is 375, versus 150 in the streptavidin plate. Due to the approach to a final value at high levels of enzyme, this difference is due to the amount of capture antibody on the plate. The silver plate binds more functional capture antibody, and thus is able to bind more enzyme when excess enzyme is present. Assuming that each antibody captures an average of 1.5 enzyme molecules, it is possible to estimate that the silver plate has approximately twice as many biotinylated antibody binding sites available. The ultimate detection limits of both plates is the same, however, and approaches femtomolar levels. This is the limit of the enzyme/substrate detection system being used.

TABLE IV

Silver Plate Immunoassay Data

Dilution Number	HRPO (mol/well)	Test 1		Test 2		Test 3		Test 1 Normalized	Test 2 Normalized	Test 3 Normalized	Average
		Antibody	Control	Antibody	Control	Antibody	Control				
1	2.73E-11	2.83E+02	2.52E+01	2.38E+02	1.83E+01	2.90E+02	2.55E+01	2.58E+02	2.20E+02	2.65E+02	2.47E+02
2	1.37E-11	3.87E+02	1.58E+01	3.46E+02	1.15E+01	3.94E+02	1.73E+01	3.71E+02	3.34E+02	3.76E+02	3.61E+02
4	6.83E-12	3.67E+02	9.82E+00	3.76E+02	7.55E+00	3.81E+02	1.22E+01	3.58E+02	3.68E+02	3.69E+02	3.65E+02
8	3.41E-12	3.36E+02	6.04E+00	3.44E+02	4.20E+00	3.63E+02	8.71E+00	3.30E+02	3.40E+02	3.54E+02	3.41E+02
16	1.71E-12	3.07E+02	5.74E+00	2.99E+02	3.37E+00	3.43E+02	7.77E+00	3.01E+02	2.96E+02	3.36E+02	3.11E+02
32	8.53E-13	2.67E+02	1.50E+01	2.70E+02	1.77E+00	2.90E+02	7.32E+00	2.52E+02	2.68E+02	2.83E+02	2.68E+02
64	4.27E-13	2.18E+02	2.83E+00	2.16E+02	2.11E+00	2.28E+02	5.53E+00	2.15E+02	2.14E+02	2.22E+02	2.17E+02
128	2.13E-13	1.61E+02	1.49E+00	1.53E+02	2.30E+00	1.50E+02	7.92E+00	1.60+02	1.51E+02	1.42E+02	1.51E+02
256	1.07E-13	7.38E+01	1.04E+00	7.18E+01	4.99E+00	7.39E+01	2.02E+00	7.27E+01	6.68E+01	7.18E+01	7.05E+01
512	5.33E-14	5.20E+01	1.35E+00	4.73E+01	2.57E+00	5.37E+01	2.32E+00	5.06E+01	4.48E+01	5.14E+01	4.89E+01
1024	2.67E-14	2.81E+01	1.49E+00	2.33E+01	4.83E+00	2.75E+01	3.26E+00	2.67E+01	1.85E+01	2.43E+01	2.31+01
2048	1.33E-14	1.20E+01	1.96E+00	1.34E+01	5.77E+00	1.43E+01	4.13E+00	1.00E+01	7.68E+00	1.02E+01	9.30E+00
4096	6.67E-15	6.66E+00	1.91E+00	1.05E+01	8.35E+00	8.61E+00	5.30E+00	4.75E+00	2.15E+00	3.31E+00	3.41E+00
8192	3.33E-15	4.73E+00	1.49E+00	1.03E+01	8.84E+00	3.03E+00	4.65E+00	3.24E+00	1.43E+00	-1.62E+00	1.02E+00
16384	1.67E-15	2.93E+00	2.41E+00	7.07E+00	6.65E+00	6.78E+00	6.25E+00	5.22E-01	4.28E-01	5.25E-01	4.92E-01
32768	8.33E-16	2.12E+00	3.86E+00	5.24E+00	5.19E+00	6.95E+00	8.48E+00	-1.74E+00	4.82E-02	-1.53E+00	-1.07E+00

TABLE V

Streptavidin Plate Immunoassay Data

Dilution Number	HRPO (mol/well)	Test 1		Test 2		Test 3		Test 1 Normalized	Test 2 Normalized	Test 3 Normalized	Average
		Antibody	Control	Antibody	Control	Antibody	Control				
1	2.73E-11	1.36E+02	6.50E+00	1.33E+02	8.55E+00	1.29E+02	5.50E+00	1.29E+02	1.24E+02	1.24E+02	1.26E+02
2	1.37E-11	1.74E+02	8.61E+00	1.24E+02	3.46E+00	1.75E+02	7.26E+00	1.65E+02	1.20E+02	1.68E+02	1.51E+02
4	6.83E-12	1.74E+02	1.96E+00	1.51E+02	2.29E+00	1.49E+02	2.06E+01	1.72E+02	1.49E+02	1.28E+02	1.50E+02
8	3.41E-12	1.62E+02	1.47E-01	1.34E+02	1.02E+00	1.38E+02	4.83E-01	1.62E+02	1.33E+02	1.38E+02	1.44E+02
16	1.71E-12	1.62E+02	-2.36E-01	1.40E+02	3.03E-02	1.30E+02	-2.53E-01	1.62E+02	1.40E+02	1.30E+02	1.44E+02
32	8.53E-13	1.49E+02	-8.89E-01	1.16E+02	6.89E-01	1.31E+02	-1.41E+00	1.50E+02	1.16E+02	1.33E+02	1.33E+02
64	4.27E-13	1.23E+02	-6.99E-01	1.28E+02	3.23E-01	1.05E+02	-1.26E+00	1.24E+02	1.28E+02	1.06E+02	1.19E+02
128	2.13E-13	1.04E+02	-1.47E-01	1.01E+02	-8.88E-01	8.99E+01	-2.05E+00	1.04E+02	1.02E+02	9.20E+01	9.93E+01
256	1.07E-13	3.86E+01	8.42E-01	5.01E+01	1.06E+00	4.32E+01	5.47E-01	3.78E+01	4.91E+01	4.26E+01	4.32E+01
512	5.33E-14	3.95E+01	1.37E+00	3.31E+01	7.59E-02	4.04E+01	6.97E-02	3.81E+01	3.31E+01	4.03E+01	3.72E+01
1024	2.67E-14	2.45E+01	1.38E+00	2.02E+01	-1.67E-01	2.45E+01	5.45E-01	2.31E+01	2.03E+01	2.40E+01	2.25E+01
2048	1.33E-14	1.29E+01	6.25E-01	9.56E+00	-8.19E-01	1.22E+01	1.06E-01	1.23E+01	1.04E+01	1.21E+01	1.16E+01
4096	6.67E-15	5.97E+00	-3.87E-01	4.76E+00	-1.42E+00	6.07E+00	-3.69E-01	6.36E+00	6.18E+00	6.44E+00	6.33E+00
8192	3.33E-15	2.75E+00	-3.01E-01	1.47E+00	-2.08E+00	3.18E+00	-6.08E-02	3.05E+00	3.55E+00	3.24E+00	3.28E+00
16384	1.67E-15	1.25E+00	-9.64E-01	1.64E-01	-1.36E+00	-2.02E+00	-9.85E-01	2.21E+00	1.52E+00	-1.04E+00	8.97E-01
32768	8.33E-16	1.07E+00	-1.22E+00	-2.82E-01	-1.87E+00	-9.76E-01	-2.00E+00	2.29E+00	1.58E+00	1.02E+00	1.63E+00

Claims

1. A bioassay plate having silver ions immobilized thereon.

2. The bioassay plate of Claim 1 wherein said plate is a polystyrene plate.

3. The bioassay plate of Claim 2 wherein said plate is a multi-well plate.

4. The bioassay plate of Claim 2 wherein said plate is a 96-well microplate.

5. A multi-well bioassay plate having silver ions immobilized thereon made by a method comprising:

a) functionalizing a multi-well bioassay plate to provide an amine - containing bioassay plate;

b) adding a polymerized glutaraldehyde to the wells of said plate and maintaining for a time and under conditions to provide a glutaraldehyde - activated bioassay plate;

c) rinsing said plate with an aqueous solution;

d) adding thiourea to the wells of said plate and maintaining for a time and under conditions whereby the thiourea reacts with the glutaraldehyde moiety of said glutaraldehyde-activated bioassay plate;

e) rinsing said plate with an aqueous solution; and

f) contacting said plate with silver ions for a time sufficient to immobilize said silver ions on said plate.

6. The multi-well bioassay plate of Claim 5 wherein said polymerized glutaraldehyde is prepared by allowing 25 wt % glutaraldehyde to polymerize at about 70°C for about 24 hours.

7. The multi-well bioassay plate of Claim 5 wherein said polymerized glutaraldehyde is maintained in the wells of said plate at about 37°C for about 24 hours.

8. The multi-well bioassay plate of Claim 5 wherein said thiourea is maintained in the wells of said plate at about 37°C for about 24 hours.

9. The multi-well bioassay plate of Claim 5 wherein said silver ions are added to said plate in the form of silver nitrate.

10. A method of making a multi-well bioassay plate having silver ions immobilized thereon comprising the steps of:

a) functionalizing a multi-well bioassay plate to provide an amine-containing bioassay plate;

b) adding a polymerized glutaraldehyde to the wells of said plate and maintaining for a time and under conditions to provide a glutaraldehyde - activated bioassay plate;

c) rinsing said plate with an aqueous solution;

d) adding thiourea to the wells of said plate and maintaining for a time and under conditions whereby the thiourea reacts with the glutaraldehyde moiety of said glutaraldehyde activated bioassay plate;

e) rinsing said plate with an aqueous solution; and

f) contacting said plate with silver ions for a time sufficient to immobilize said silver ions on said plate.

11. The method of Claim 10 wherein said polymerized glutaraldehyde is prepared by allowing 25 wt % glutaraldehyde to polymerize at about 70°C for about 24 hours.

12. The method of Claim 10 wherein said polymerized glutaraldehyde is maintained in the wells of said plate at about 37°C for about 24 hours.

13. The method of Claim 10 wherein said thiourea is maintained in the wells of said plate at about 37°C for about 24 hours.

14. The method of Claim 10 wherein said silver ions are added to said plate in the form of silver nitrate.

15. A method for detecting an antigen comprising the steps of:

a) incubating a multi-well bioassay plate having silver ions immobilized thereon with a biotinylated antibody having specificity for said antigen to provide a bioassay plate having said antibody immobilized thereon;

b) incubating said plate with a solution containing said antigen;

c) washing said plate with an aqueous solution;

d) incubating said plate with a labeled antibody having specificity for said antigen;

e) washing said plate with an aqueous solution; and

f) detecting said label, wherein detection of said label is indicative of the presence of said antigen.

16. A method for detecting a first antibody comprising the steps of:

a) incubating a multi-well bioassay plate having silver ions immobilized thereon with a biotinylated antigen that is reactive with said first antibody to provide a bioassay plate having said antigen immobilized thereon;

b) incubating said plate with an aqueous solution containing said first antibody;

c) washing said plate with an aqueous solution;

d) incubating said plate with an aqueous solution containing a labeled second antibody that binds to said first antibody;

e) washing said plate with an aqueous solution; and

f) detecting said label, wherein detection of said label is indicative of the presence of said first antibody.

17. A kit for the detection of a first antibody comprising a first container containing a bioassay plate having silver ions immobilized thereon.

18. The kit of Claim 17 further comprising a second container containing a biotinylated antigen that is reactive with said first antibody.

19. The kit of Claim 18 further comprising a third container containing a labeled second antibody that binds to said first antibody.

20. A kit for the detection of an antigen comprising a first container containing a bioassay plate having silver ions immobilized thereon.

21. The kit of Claim 20 further comprising a second container containing a biotinylated antibody having specificity for said antigen.

22. The kit of Claim 21 further comprising a third container containing an antibody having specificity for said antigen.

23. An apparatus for activating microplates comprising:

a) a housing;

b) a reagent addition/withdrawal chamber disposed in said housing, said reagent addition/withdrawal chamber including reagent and wash storage containers in communication with a manifold, said manifold in communication with dispense lines disposed to deliver wash and reagent to a microplate, and further including aspirate lines in communication with the manifold, said manifold in communication with a waste container, said aspirate lines disposed to aspirate spent reagent from said microplate;

c) an incubation chamber disposed in said housing, said incubation chamber including a means for vertically delivering a non-reactive sealing plate to said microplate, and a means for heating and agitating said microplate.

d) a means for horizontally conveying a microplate into and out of said addition/withdrawal chamber and between said addition/withdrawal chamber and said incubation chamber.

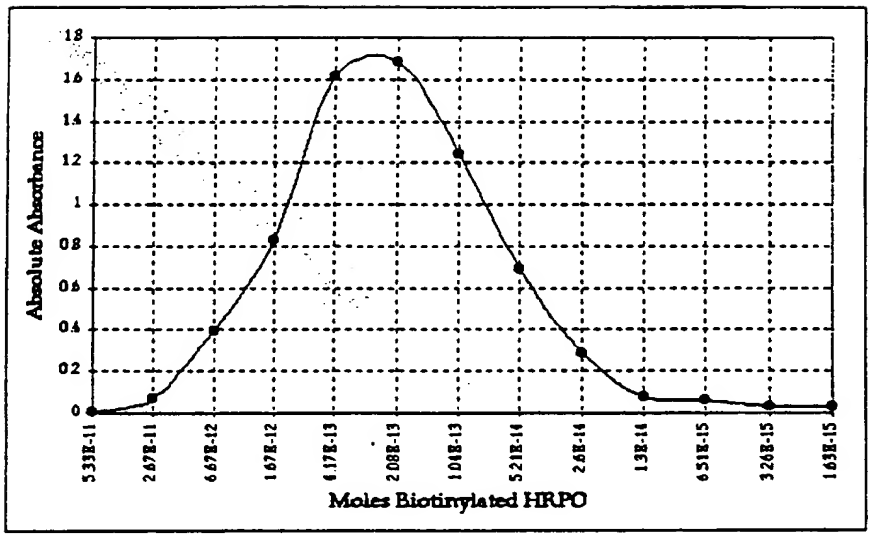
Absolute Absorbance
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5.33E-11
2.67E-11
6.67E-12
1.67E-12
4.17E-13
2.08E-13
1.04E-13
5.21E-14
2.6E-14
1.3E-14
6.51E-15
3.26E-15
1.63E-15
Moles Biotinylated HRPO

FIG. 1

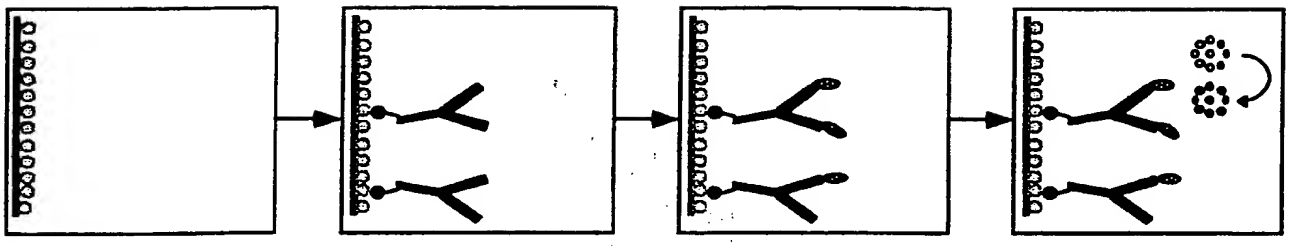

FIG. 2

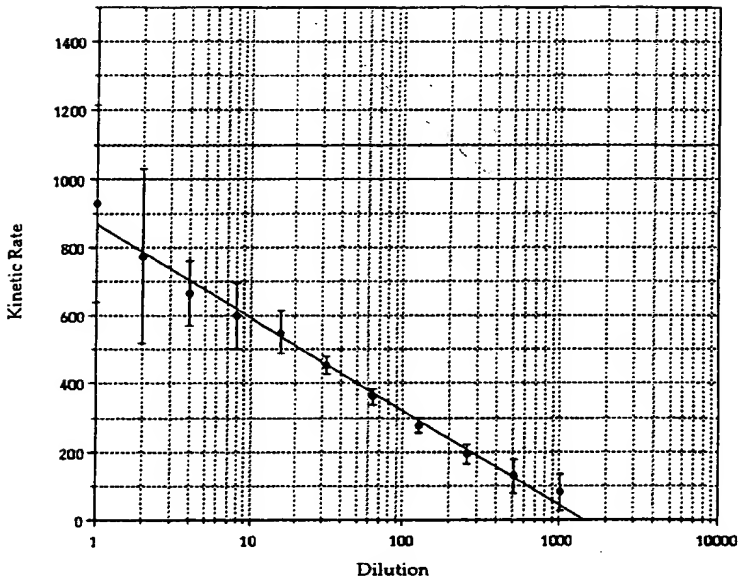
Kinetic Rate
1400
1200
1000
800
600
400
200
0
1
10
100
1000
10000
Dilution

FIG. 3

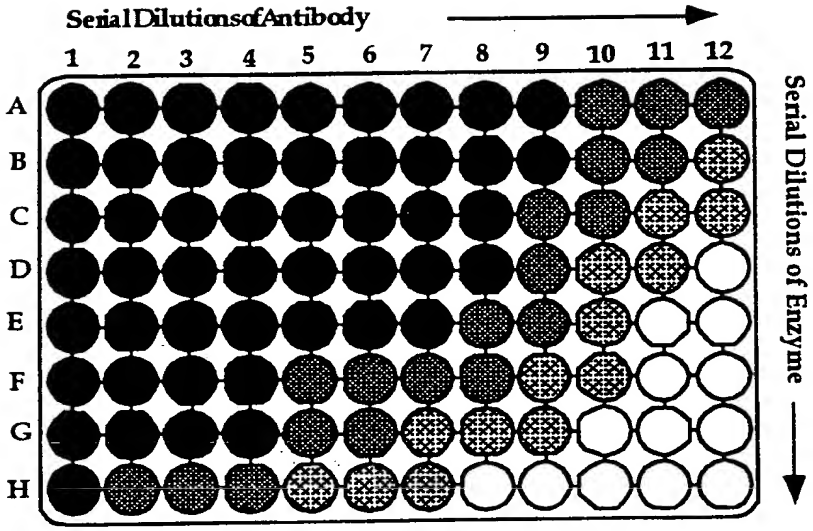
Serial Dilutions of Antibody
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
Serial Dilutions of Enzyme

FIG. 4

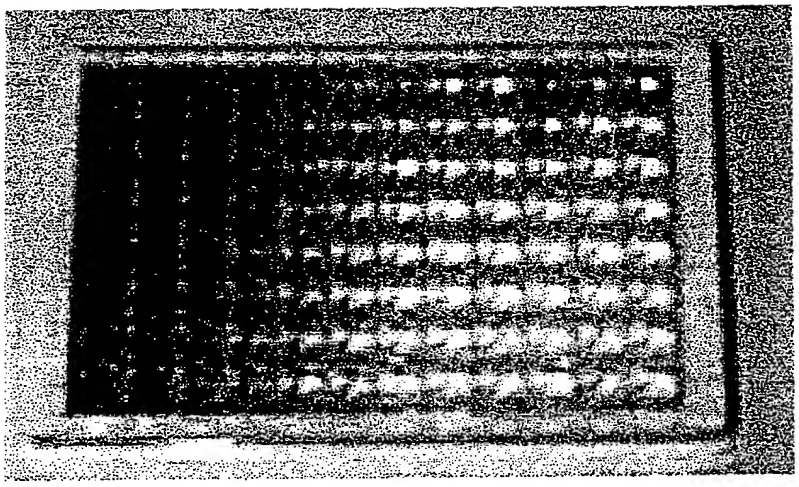

FIG. 5

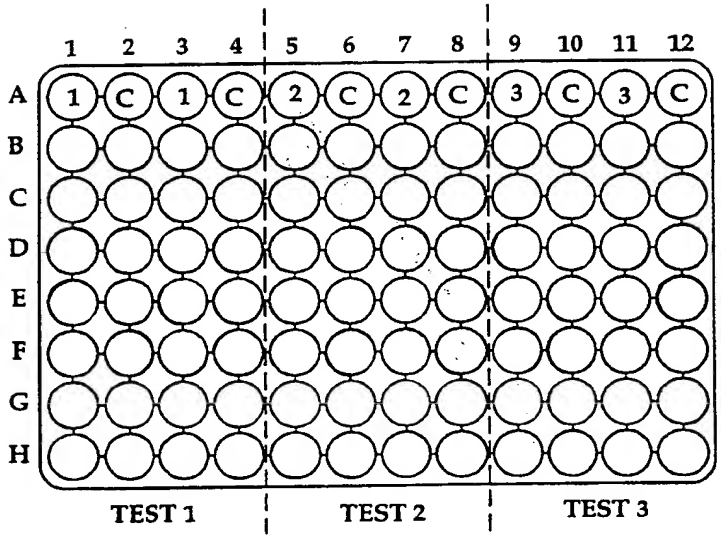
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
1 C 1 C 2 C 2 C 3 C 3 C
TEST 1
TEST 2
TEST 3

FIG. 6

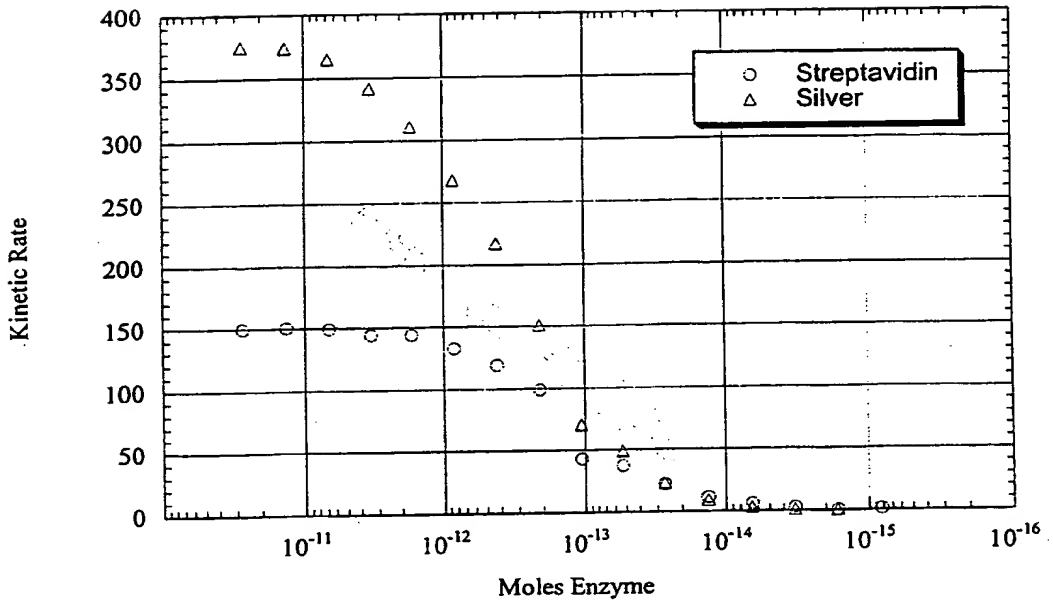
400
350
300
250
200
150
100
50
0
Kinetic Rate
Streptavidin
Silver
10^{-11}
10^{-12}
10^{-13}
10^{-14}
10^{-15}
10^{-16}
Moles Enzyme

FIG. 7

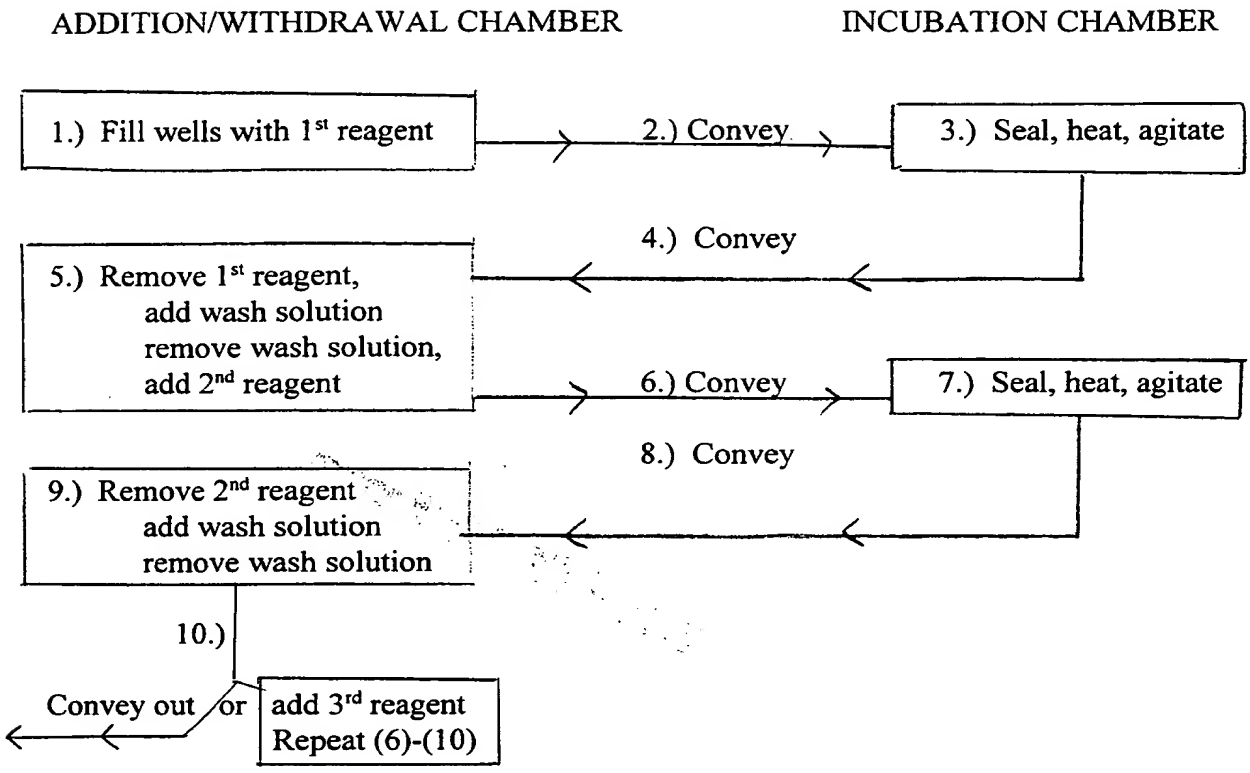
ADDITION/WITHDRAWAL CHAMBER
INCUBATION CHAMBER
1.) Fill wells with 1st reagent
2.) Convey
3.) Seal, heat, agitate
4.) Convey
5.) Remove 1st reagent,
add wash solution
remove wash solution,
add 2nd reagent
6.) Convey
7.) Seal, heat, agitate
8.) Convey
9.) Remove 2nd reagent
add wash solution
remove wash solution
10.)
Convey out
or
add 3rd reagent
Repeat (6)-(10)

FIG. 8

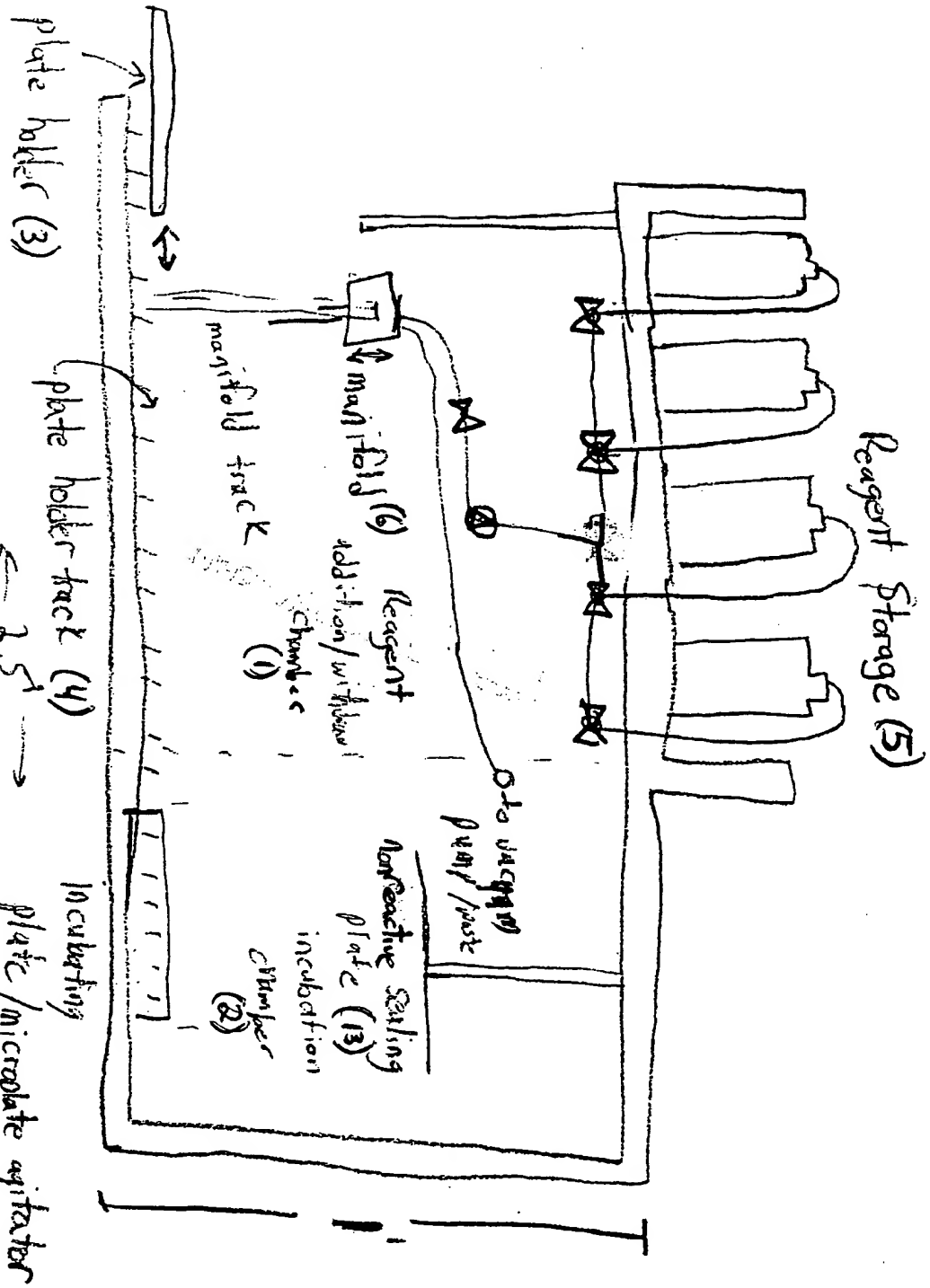
Reagent Storage (5)
To vacuum pump/waste
Nonreactive sealing plate (13)
incubation chamber (12)
Reagent addition/withdrawal chamber (11)
manifold (6)
manifold track
plate holder (3)
plate holder track (4)
incubating plate/microplate agitator (14)

FIG. 9

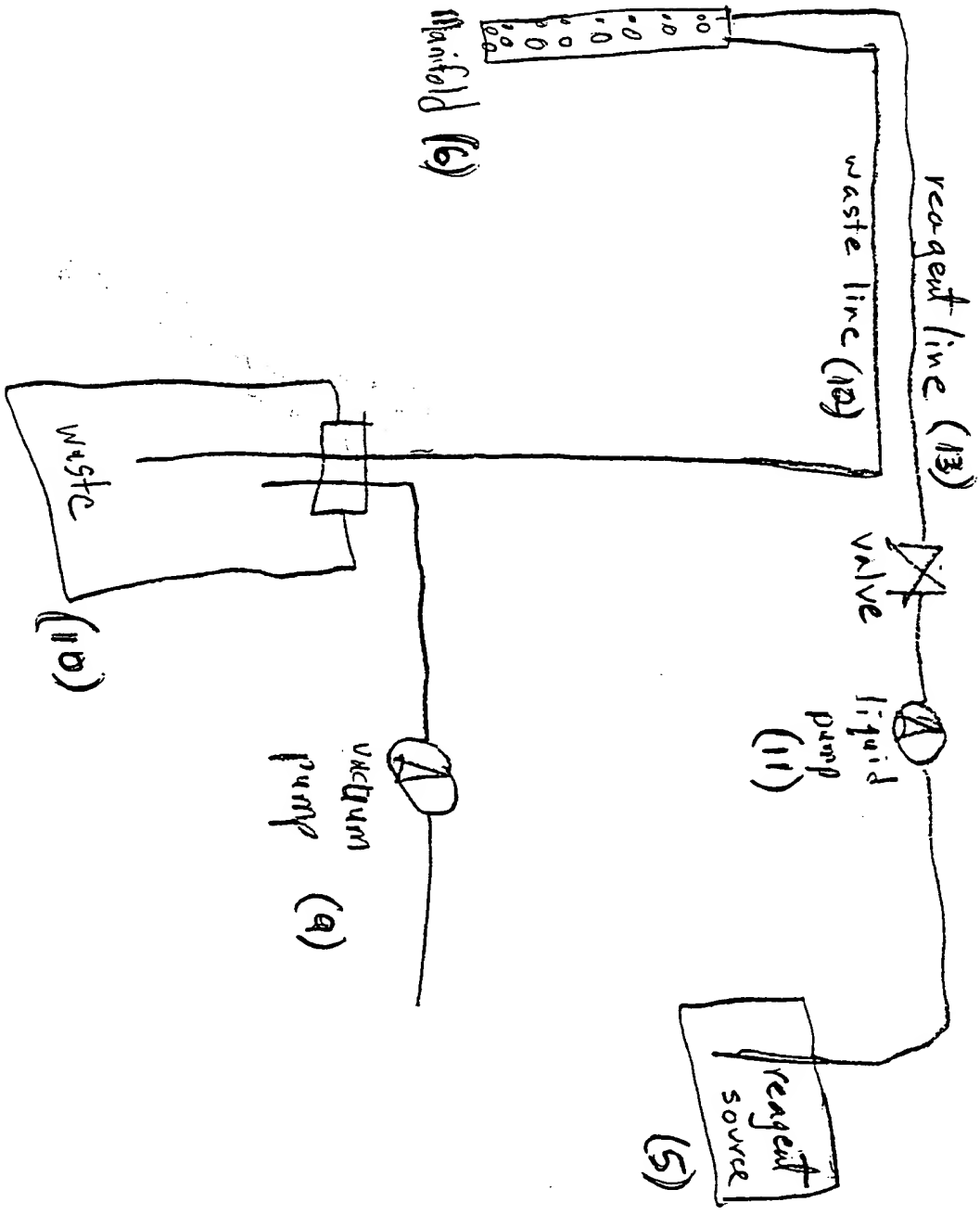
Manifold (6)
waste line (12)
reagent line (13)
valve
liquid pump (11)
reagent source (5)
waste (10)
vacuum pump (9)

FIG. 10

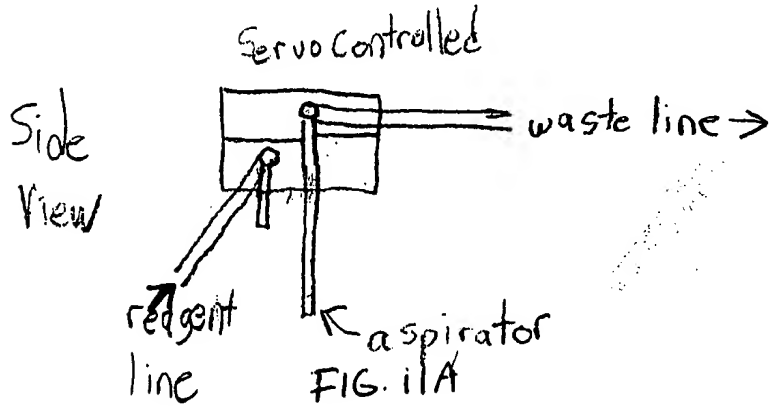

FIG. 11A

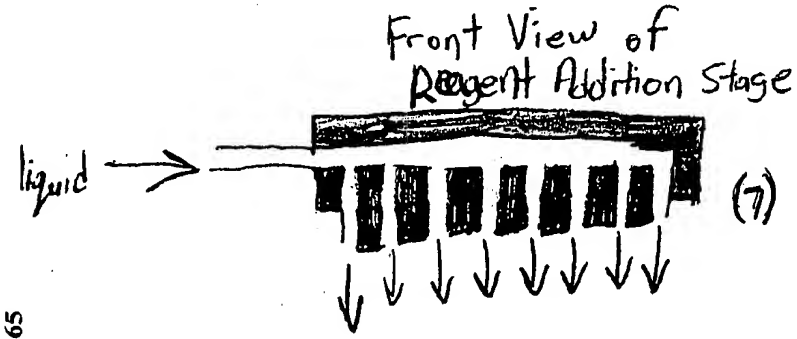

FIG. 11B

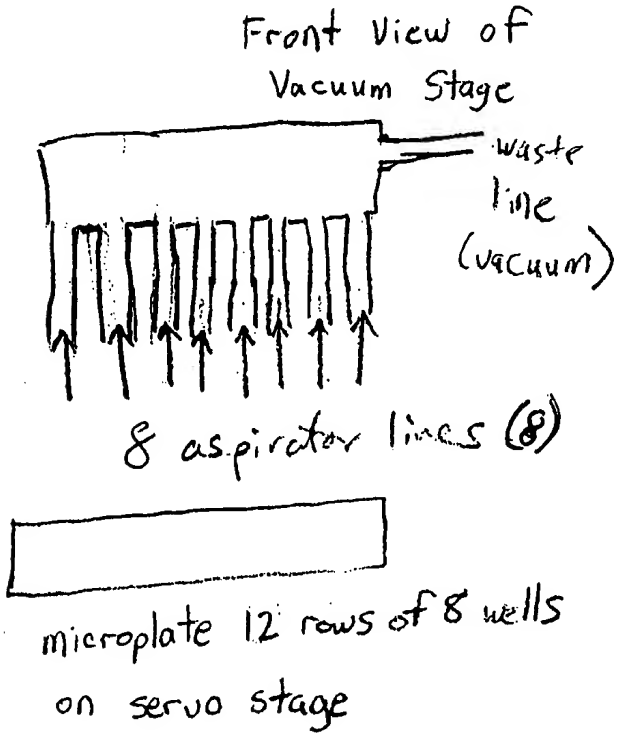

FIG. 11C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23902

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :B01L 3/00; C12Q 1/68; B05D 5/12; G01N 33/53, 33/553, 33/549
US CL :422/99, 102; 427/123, 125; 435/6, 7.1; 436/525, 532

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/99, 102; 427/123, 125, 384, 389, 404, 414; 435/6, 7.1, 7.5, 7.6; 436/518, 524, 525, 531, 532, 808, 809

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,609,907 A (NATAN) 11 March 1997, col. 2, lines 60-67.	1 --- 2
Y	US 5,552,086 A (SIIMAN et al) 03 September 1996, col. 1, lines 49-50 and 64-65, col. 8, line 1, and col. 10, lines 64-67.	2, 5, 10
X --- Y	US 5,814,516 A (VO-DINH) 29 September 1998, columns 7 and 8.	1 --- 2, 5, 10
X --- Y	US 5,605,798 A (KOSTER) 25 February 1997, column 7, lines 54-65.	1 --- 3,4

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,705,813 A (APFFEL et al) 04 January1988, column 6, lines 4-20, column 7, lines 1-5, column 8, lines 22-31.	1-3, 15, 16
Y	US 4,775,636 A (MOEREMANS et al) 04 October 1988, column 3, lines 25-60, column 4, lines 7-50, column 6, lines 30-50.	1-3, 14-16
Y	US 5,543,332 A (LIHME et al) 06 August 1996, columns 2 and 3, also column 6.	15, 16
Y, P	US 5,908,746 A (SUZUKI et al) 01 JUNE 1999, column 1, lines 37-50, column 2, lines 29-31 and lines 56-57, column 5, lines 5-35, column 8, lines 19-45.	2-5, 10, 15, 16
Y, E	US 5,972,615 A (AN et al) 26 October 1999, column 7, lines 5-21, column 27, lines 48-52, column 28, lines 52-66, columns30-31.	2, 3, 15-22

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(54) Title: IMMOBILIZED SILVER IMMUNOASSAY SYSTEM

(57) Abstract

The present invention provides bioassay plates having silver ions immobilized thereon, which are useful in immunoassays for detection of antibodies or antigens. Methods of making and using the biossay plates are also provided, as well as an apparatus for making activated bioassay plates.

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IMMOBILIZED SILVER IMMUNOASSAY SYSTEM

Specification

Background of the Invention

Since the late 1950s, when the work of Rosalyn Yalow and Solomon Berson (Yalow et al., 1959, Nature (London) 184:1648; Yalow et al., 1960, J. Clin. Invest 39:1157) first illuminated the possibilities of immunoassays, immunodiagnostic techniques based on the specific interaction of antibody and antigen have become of paramount importance in the clinical, agricultural, food, veterinary, and environmental sectors. In 1996, it was estimated that the worldwide market of immunoassay products was $10 billion in the clinical sector alone, and increasing at an annual rate of 10% (Deshpande, Enzyme Immunoassay: From Concept to Product Development, New York:Chapman & Hall, 1996). This market is driven by an ever-increasing desire for assays of greater sensitivity and specificity, at reasonable financial costs. It is in this environment that the specific and irreversible interaction between avidin or streptavidin and biotin has found use.

Streptavidin, a close relative of egg white avidin, is expressed in *Streptomyces avidinii* (Green, 1990, Methods in Enzymology 184:51), and both avidin and streptavidin exhibit an affinity for biotin on the order of 10^{15} M^{-1}. The streptavidin-biotin system has become a widely-used tool of molecular biology in such applications as affinity chromatography, cytometry, nucleic acid research, and diagnostics (Diamandis et al., 1991, Clin. Chem. 37:625; Wilchek et al., 1988, Anal. Bio Chem. 171:1). A common immunological procedure calls for the use of streptavidin-coated microtiter plates, which are used to capture either biotinylated antibodies or antigens. Since the assay is based on the interaction of streptavidin and biotin, universal kit-based assay formats are possible. These universal assays are also the basis of many automated immunological testing systems (Chan, ed., 1996, Immunoassay Automation: An Updated Guide to Systems, San Diego: Academic Press, 51-308). A format utilizing a microtiter-based enzyme-linked immunosorbent

assay (ELISA) can measure a wide variety of analytes using a dual antibody or "sandwich" immunoassay. The choice of a streptavidin-coated solid support is made to overcome the limitations present in the direct antibody coating of polystyrene supports, which can result in unreliable or nonuniform coating of the solid support, in addition to the steric affects of binding upon the antibody. However, while streptavidin systems allow for universal ELISA kits and can improve assay sensitivity, coated plates can be costly.

Research examining the behavior of biotin has found that immobilized silver ions will bind biotinylated compounds both strongly and, in this case, reversibly. This research has been done with both immobilized metal affinity chromatography (IMAC) (Garcia et al., 1994, Reactive Polymers 23:249; Kim et al., 1995, Art. Cells, Blood Subs., and Immob. Biotech. 23:555; Miles et al., 1995, J. Chromatogr. A. 702:173) and paramagnetic particles (Ramirez-Vick, 1997, Ph.D. Dissertation, Arizona State University, Tempe, AZ). It has been discovered in accordance with the present invention that immobilized silver ions can be used in an immunoassay format to provide a sensitive and inexpensive universal assay.

Summary of the Invention

The present invention provides an immunoassay system comprising bioassay plates having silver immobilized thereon. The present invention further provides a method of making bioassay plates having silver immobilized thereon.

In another embodiment, the present invention provides a method for detecting an antigen or antibody, and a kit useful for the detection of an antigen or antibody.

An apparatus for providing activated bioassay plates is also provided by the present invention.

Brief Description of the Drawings

Fig. 1 is a graph depicting the sensitivity of a microtiter plate having silver immobilized thereon.

Fig. 2 is a schematic of an immunoassay utilizing microtiter plates having silver immobilized thereon, and biotinylated capture antibodies.

Fig. 3 is a graph providing a kinetic analysis of a horseradish peroxidase immunoassay.

Fig. 4 is a schematic of a checkerboard assay.

Fig. 5 shows the results of a checkerboard assay.

Fig. 6 is a schematic depicting the arrangement of activated and control wells of a microtiter plate.

Fig. 7 is a graph of immunoassay results comparing a streptavidin method with the silver method of the present invention.

Fig. 8 is a flow diagram describing the apparatus of the present invention.

Fig. 9 is a side view of the apparatus of the invention.

Fig. 10 is a diagram of the liquid handling system of the invention.

Figs. 11a-11c are diagrams of the liquid transfer manifold of the invention. Fig. 11a is a side view of the reagent addition stage and vacuum stage; Fig. 11b is a front view of the reagent addition stage; Fig. 11c is a front view of the vacuum stage.

Detailed Description of the Invention

The present invention provides an immunoassay system comprising bioassay plates having silver, in particular silver ions, immobilized thereon. The invention further provides methods of making and using such bioassay plates. The bioassay plates and immunoassay of the present invention are useful for the detection of antibodies and antigens, and provide cost and sensitivity advantages relative to the streptavidin-coated bioassay plates of the prior art.

The bioassay plates used in the present invention are microwell, or microtiter, plates known in the art for immunoassays, and are commercially available. Conventional microwell plates are 96-well microplates having wells arranged on an 8 x 12 matrix on 9 mm centers. Each well holds approximately 300 microliters. 384-well plates are also available, in which the wells are arranged in a 16 x 24 matrix on a 4.5 mm center, with each wells having a brim volume of approximately 80 microliters. Well plates defined by larger matrices, e.g. 1536 well plates, are also available. The number and configuration of the wells are not critical to the present

invention, and are used by way of example only. The bioassay plates used in accordance with the present invention are plastic, and preferably polystyrene.

Bioassay plates having silver ions immobilized thereon are made by a method comprising functionalizing a multi-well bioassay plate to provide an amine-containing bioassay plate, adding polymerized glutaraldehyde to the wells of the plate for a time and under conditions whereby the amines are activated by glutaraldehyde, rinsing the plates with an aqueous solution, adding thiourea to the wells of the plate for a time and under conditions whereby the thiourea is reacted with a glutaraldehyde moiety of the glutaraldehyde-activated bioassay plate, rinsing the plate with an aqueous solution, and contacting the plate with silver ions for a time and under conditions whereby the silver ions are immobilized on said plate.

The bioassay plate may be constructed of any material that can be functionalized to contain an amine group. In a preferred embodiment, the multi-well bioassay plate is a plastic multi-well bioassay plate. For example, the plate may be made of polystyrene, polyethylene, polypropylene, or other primary polymers or composite resins. Polystyrene is particularly preferred. Methods for functionalizing these materials to contain an amine group are known in the art. For example, a polystyrene bioassay plate can be functionalized to contain an amine group by methods known in the art and disclosed for example in Immobilized Affinity Ligand Techniques, Hermanson et al., eds., San Diego: Academic Press, 1992, the disclosure of which is incorporated herein by reference. Aminated polystyrene bioassay plates are also commercially available, for example from Corning (Corning, NY), NUNC (Denmark) and Micro Membranes (Newark, NJ). Preferably the plate is amidated or aminated to contain from about 1×10^{13} to 1×10^{14} amine sites per cm^2.

Polymerized glutaraldehyde may be prepared by allowing glutaraldehyde (25 wt %) to polymerize, for example for from 1 to 36 hours at from 23°C to 70°C. In a preferred embodiment polymerization is at 70°C for about 24 hours. The polymerized glutaraldehyde is added to the wells of the plate and incubated under conditions whereby a glutaraldehyde-activated plate is produced, for example for from 1 to 36 hours at from 23°C to 70°C. In a preferred embodiment, incubation is at 35 to 50°C for 1 to 24 hours, and more preferably at about 37°C for about 24 hours. The

plate is rinsed with an aqueous solution, for example deionized water, to remove unreacted glutaraldehyde. The wells of the plate are then filled with thiourea, for example from 0.01M to 1M solution, and preferably a 1M solution, under conditions suitable for reaction with the glutaraldehyde moiety, for example for from 1 to 36 hours at from 23°C to 70°C. In a preferred embodiment at, incubation is for 1 to 24 hours at 35 to 50°C, and more preferably about 24 hours at about 37°C. The plate is rinsed with an aqueous solution, for example deionized water, to remove unreacted thiourea.

Silver ions, preferably in the form of silver nitrate, are then added to the plate under conditions whereby silver ions are immobilized on the plate, for example for from 1 to 36 hours at from 23°C to 70°C. In a preferred embodiment, incubation is for about 24 hours at about 37°C. The plates are then rinsed with an aqueous solution, for example deionized water, and may be stored until use, preferably in an opaque sleeve.

The bioassay plates having silver ions immobilized thereon are useful in a method for detecting an antigen or an antibody. It has been discovered in accordance with the present invention that the silver ions immobilized on bioassay plates are capable of strong binding to biotinylated antibodies and antigens. Accordingly, the plates of the invention may be used in standard enzyme-linked immunosorbent assays (ELISAs). For example, a bioassay plate having silver ions immobilized thereon is incubated with biotinylated antibody to provide a bioassay plate having the antibody immobilized thereon. After washing with an aqueous solution, the plate is incubated with a solution containing the cognate antigen under conditions whereby the antigen binds to the immobilized antibody, followed by another washing step. The antigen is then detected, for example by subsequent incubation with a labeled antibody having specificity for the antigen. Detectable labels for antibodies are known in the art and include radiolabels, fluorescent tags, and enzyme conjugates. In a preferred embodiment, the aqueous solution contains deionized water and Tween (polyoxyethylene sorbitan monolaurate).

An antibody may be detected using the plates of the present invention in an indirect ELISA assay. For example, a bioassay plate having silver ions immobilized

thereon is incubated with a biotinylated antigen to provide a bioassay plate having the antigen immobilized thereon. After washing with an aqueous solution, the plate is incubated with a solution containing the cognate primary antibody under conditions whereby said antibody binds to the immobilized antigen. After incubation and washing, a labeled secondary antibody is added and incubated under conditions whereby it binds to the primary antibody. After washing, the secondary antibody is detected, wherein detection thereof indicates the presence of the primary antibody. Detectable labels for antigens are known in the art and include radiolabels, fluorescent tags, and enzyme conjugates. In a preferred embodiment, the aqueous solution contains deionized water and Tween.

Conditions for biotinylating antibodies and antigens are well known in the art and disclosed, for example, by Bayer et al., "Protein Biotinylation" (1990) Methods in Enzymology 184:138 and O'Shannessy "Antibodies Biotinylated via Sugar Moieties" (1990) Methods in Enzymology 184:162, the disclosures of which are incorporated herein by reference. Conditions for performing ELISAs are well-known in the art and disclosed, for example, by Harlowe, et al., (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, the disclosure of which is incorporated herein by reference.

The present invention further provides a kit useful for the detection of an antigen or antibody. The kit comprises, in a first container, a bioassay plate having silver ions immobilized thereon. In a preferred embodiment, the bioassay plate is a polystyrene multi-well plate. The kit may optionally contain a second container containing a biotinylated antibody or a biotinylated antigen. The kit may optionally contain a third container containing labeled antibody, when the second container contains a biotinylated antibody, or a labeled secondary antibody, when the second container contains a biotinylated antigen.

The present invention further provides an apparatus useful for the automated production of microplates having modified surface chemistry. As shown by the flow diagram in Fig. 8, the apparatus provides for filling the wells of a microplate with a reagent in an addition/withdrawal chamber; conveying the microplate to an incubation chamber in which the microplate is sealed, heated and agitated, and unsealed;

conveying the microplate to the addition/withdrawal chamber for evacuation of reagent, washing, and addition of a second reagent; conveying the microplate to the incubation chamber for sealing, heating and agitation, and unsealing; conveying the microplate to the addition/withdrawal chamber for evacuation of reagent and washing; followed by subsequent cycles of reagent addition and incubation, or conveyance of the microplate out of the machine.

In a preferred embodiment, and with reference to Figs. 9, 10, 11a, 11b and 11c, the apparatus comprises a housing having disposed therein a reagent addition/withdrawal chamber (1) and an incubation chamber (2). Microplates are conveyed into and between the chambers by means of a plate holder (3) movable horizontally by a plate holder track (4). Reagents and wash solution are provided in storage containers (5) connected by reagent lines (13) to the dispense portion of a manifold (6) which delivers reagent and wash solution by dispense lines (7) by means of a liquid pump (11). After reagent addition, microplates positioned on the plate holder (3) are conveyed via the plate holder track (4) into the incubation chamber (2). The microplate is sealed by a non-reactive sealing plate (13) delivered vertically. The incubation chamber further provides a means for heating and agitating the microplate (14). After a time predetermined by the user, the microplate is conveyed to the reagent addition/withdrawal chamber (1) via plate holder track (4). Spent reagent is removed through aspirator lines (8) and withdrawn by the aspirate portion of the same manifold (6) by means of a vacuum pump (9) through waste lines (12) to a waste container (10). Wash solution is added through the dispense portion of the manifold (6) which delivers wash solution through dispense lines (7). Wash solution is removed through aspirator lines (8) and withdrawn by the aspirate portion of manifold (6) by means of a vacuum pump (9) through waste lines (12) to a waste container (10).

The foregoing steps are carried out in an automatic programmed manner under the control of electronic circuitry contained in the housing.

All references cited herein are incorporated in their entirety.

The following examples serve to further illustrate the present invention.

Example 1

A polystyrene 96 well microtiter plate aminated to provide approximately 2 x 10^{13} active amine sites per cm^2 was obtained from Corning (Corning, NY). Glutaraldehyde (25 wt %) which had been allowed to polymerize at 70°C for 24 hours was added to each well of the microplate, which was then incubated at 37°C for 24 hours to facilitate plate activation. The plate was then rinsed with deionized water and wells filled with a 1M solution of thiourea, followed by an additional 24 hour period of incubation at 37°C. After another rinsing of the plate, a 1M solution of silver nitrate was allowed to contact the plate during another 24 hour incubation at 37°C. The plate was then rinsed extensively.

The biotin-binding capability of such an immobilized silver microtiter plate was tested as follows. A complete plate was assembled from stripwells, using alternating strips of unactivated and silver-containing wells, where the silver-containing strips were in the odd-numbered rows and the unactivated strips were in the even-numbered rows. The test consisted of the binding of biotinylated horseradish peroxidase (bHRPO), which was detected using the chromogenic reaction of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS®). This was a one-step, unamplified assay; 150 µl of the bHRPO solution was added to the wells using doubling dilutions, and allowed to bind for one hour. Following this, the plates were rinsed with deionized water and the ABTS® in citrate buffer was added. The developed color in the wells was read after one hour at room temperature using a BioRad Benchmark Microplate Reader set at 415 nm. The plate setup and moles of bHRPO corresponding to each dilution are shown in Tables I and II, respectively.

TABLE I

	1	2	3	4	5	6	7	8	9	10	11	12
A	no bHRPO		no bHRPO		no bHRPO		no bHRPO		no bHRPO		no bHRPO	
B	no bHRPO		1 / 1		1 / 64		1 / 4096		1 / 262144		no bHRPO	
C	no bHRPO		1 / 2		1 / 128		1 / 8192		1 / 524288		no bHRPO	
D	no bHRPO		1 / 4		1 / 256		1 / 16384		1 / 1048576		no bHRPO	
E	no bHRPO/ABTS		1 / 8		1 / 512		1 / 32768		1 / 2097152		no bHRPO/ABTS	
F	no bHRPO/ABTS		1 / 16		1 / 1024		1 / 65536		1 / 4194304		no bHRPO/ABTS	
G	no bHRPO/ABTS		1 / 32		1 / 2048		1 / 131072		1 / 8388608		no bHRPO/ABTS	
H	no bHRPO/ABTS		no bHRPO/ABTS		no bHRPO/ABTS		no bHRPO/ABTS		no bHRPO/ABTS		no bHRPO/ABTS	

TABLE II

Dilution	Grams bHRPO	Moles bHRPO
1 / 1	2.40E-06	5.33E-11
1 / 2	1.20E-06	2.67E-11
1 / 4	6.00E-07	1.33E-11
1 / 8	3.00E-07	6.67E-12
1 / 16	1.50E-07	3.33E-12
1 / 32	7.50E-08	1.67E-12
1 / 64	3.75E-08	8.33E-13
1 / 128	1.88E-08	4.17E-13
1 / 256	9.38E-09	2.08E-13
1 / 512	4.69E-09	1.04E-13
1 / 1024	2.34E-09	5.21E-14
1 / 2048	1.17E-09	2.60E-14
1 / 4096	5.86E-10	1.30E-14
1 / 8192	2.93E-10	6.51E-15
1 / 16384	1.46E-10	3.26E-15

TABLE II

Dilution	Grams bHRPO	Moles bHRPO
1 / 32768	7.32E-11	1.63E-15
1 / 65536	3.66E-11	8.14E-16
1 / 1E+05	1.83E-11	4.07E-16
1 / 3E+05	9.16E-12	2.03E-16
1 / 5E+05	4.58E-12	1.02E-16
1 / 1E+06	2.29E-12	5.09E-17
1 / 2E+06	1.14E-12	2.54E-17
1 / 4E+06	5.72E-12	1.27E-17
1 / 8E+06	2.85E-13	6.36E-18

After applying statistical curve fitting techniques, a concentration activity curve was generated and is shown in Figure 1. Results are presented in terms of moles of bHRPO. As demonstrated therein, the detection limit of this system approaches femtomolar levels, even using an unamplified system. The typical Gaussian response curve of ELISA is also seen in Figure 1, which further demonstrates that the maximum sensitivity of this assay occurs at bHRPO levels of 2 x 10^{-13} moles.

Example II

Example I demonstrated that immobilized silver microtiter plates are capable of binding biotin and a biotinylated antigen. This example demonstrates that biotinylated capture antibodies can be bound to immobilized silver microtiter plates and used for antigen capture.

Immobilized silver microtiter plates were prepared as described in Example I. The plates were incubated with biotinylated anti-peroxidase antibodies (Jackson Immunological) at an antibody concentration of 1.2 mg/ml diluted 1:100, with addition of 150 microliters to each well for an hour. After washing the plates with 50 mM phosphate buffer with 0.1% v/v Tween 20 detergent (Phosphate/Tween buffer), the enzyme horseradish peroxidase (Sigma) was added, in the amounts shown in

Table III, for one hour incubation. Phosphate/Tween buffer was used as the dilution buffer in the assay.

The results of eight assays of the type shown in Figure 2 are presented in Figure 3, along with 2σ error limits. The data for the immunoassays is shown in Table III, using the eight simultaneous assay replications for a basic statistical analysis. Figure 3 uses kinetic rates to determine the detection of enzyme, which allows for a linear fit of the data when presented on a semi-log plot using the dilution number of the initial enzyme solution. At the initial dilutions, the margin of error is rather high, due to the high amounts of enzyme in the initial solution (2.56×10^{-11} moles per well) producing extremely rapid kinetics. As the enzyme goes through doubling dilutions, the kinetics are measured more easily and the precision of the assay improves at dilution number 16 (1.6×10^{-12} moles per well). The detection limit of this assay is in the region of dilution number 1000 (0.25 femtomoles), which approaches the theoretical detection limit of the enzyme substrate system being used. (Deshpande, Enzyme Immunoassays: From Concept to Product Development, New York, Chapman & Hall (1996), 1-422.

TABLE III

Kinetic Immunoassay Data

Dilution Number	HRPO (g/well)	HRPO (mol/well)	Kinetic Rate	2σ Limits
1	1.13E-06	2.56E-11	927.65	286.73
2	5.63E-07	1.28E-11	773.66	253.20
4	2.81E-07	6.40E-12	666.47	95.75
8	1.41E-07	3.20E-12	598.88	97.22
16	7.03E-08	1.60E-12	551.07	61.58
32	3.52E-08	8.00E-13	452.40	25.79
64	1.76E-08	4.00E-13	361.72	24.07
128	8.79E-09	2.00E-13	277.00	20.58
256	4.39E-09	1.00E-13	193.03	27.47

TABLE III

Kinetic Immunoassay Data

Dilution Number	HRPO (g/well)	HRPO (mol/well)	Kinetic Rate	2σ Limits
512	2.20E-09	5.00E-14	128.36	49.72
1024	1.10E-09	2.50E-14	80.85	53.07
2048	5.49E-10	1.25E-14	65.28	62.02
4096	2.75E-10	6.25E-15	60.26	61.08
8192	1.37E-10	3.12E-15	62.54	60.02
16384	6.87E-11	1.56E-15	52.25	78.54
32768	3.43E-11	7.81E-16	46.92	83.12
65536	1.72E-11	3.91E-16	36.93	69.52
131072	8.58E-12	1.95E-16	40.52	69.90

The basis of the foregoing is the assumption that the enzyme binds only to the antibodies, which specifically select it from the test solution. In order for this to be valid, background binding of the enzyme to the plate must be at insignificant levels. In order to determine this, a checkerboard assay was performed (Deshpande, supra). The assay arrangement is shown in Figure 4. When performed, the initial concentrations of the antibody and the enzyme were the same as described above, as were all buffers and incubation periods. The checkerboard assay, which is primarily visual, is a means to determine the effect of the antibody concentration and the enzyme concentration on the assay results. As seen in Figure 5, the enzyme only binds when antibody is present in the well. As antibody concentrations decrease horizontally across the plate, enzyme binding falls rapidly, as shown by the absence of color on the right side of the plate, even at the extreme enzyme concentrations introduced into row A.

Example III

The foregoing examples demonstrate that immobilized silver is capable of binding biotin in the bHRPO assay and that effective immobilization of capture antibodies in the immobilized silver microplate wells is possible. In the present example, the silver ion immunoassay format is then compared to the current streptavidin technology used to bind biotinylated antibodies. Plates coated with streptavidin (Xenopore, XPS 010 00) were obtained for this purpose. These plates represent the best of the currently available products since they feature a covalent linkage between the streptavidin and the plate surface, and are also blocked with a proprietary nonbiotinylated protein to inhibit background binding. No blocking agents are used on the silver plates. A side by side comparison with an immobilized silver plate prepared according to Example I was performed as follows.

Both the streptavidin plate and the silver plate were hydrated and rinsed with 50 mM pH 7 phosphate buffer. The wells in the plates were filled to capacity with buffer and allowed to stand at room temperature for 10 minutes. The plates were then rinsed twice with the same buffer.

Biotinylated anti-peroxidase antibodies (Jackson Immunological) were used to coat the plates. In this assay, 150 µl of antibody solution (1.2 mg/ml) diluted to 1:100 was added to the wells in the odd numbered columns. The even numbered columns were used as control wells, and were filled with 150 µl of buffer. This arrangement of activated and control wells is shown in Figure 6. The buffer used to dilute the antibodies and fill the control wells was 50 mM pH 7 phosphate buffer with 0.1% v/v Tween 20 Phosphate/Tween) added to inhibit any hydrophobic binding in the plates. The plates were then covered and allowed to stand at room temperature for 1.5 hours.

Both plates were thoroughly washed using a Bio-Rad Plate Washer filled with Phosphate/Tween buffer as the wash buffer. Horseradish peroxidase (Sigma) was used as the antigen in this test. A solution of peroxidase was created by adding 8 x 10^{-4}g of the enzyme to 10 ml of Phosphate/Tween. 1 ml of this solution was then added to 9 ml of buffer. 300 µl of the diluted enzyme solution was added to wells A1, A2, A5, A6, A9, and A10. The amount of enzyme added to these primary wells is shown in Table IV. The remainder of the wells were filled with 150 µl of

Phosphate/Tween. For Test 1, 150 µl of solution was withdrawn from A1 and A2 and diluted into B1 and B2. This procedure was repeated down the plate, with the solution from H1 and H2 carried to wells A3 and A4. Tests 2 and 3 were performed in a similar fashion. After all of these doubling dilutions were complete, the plates were covered and allowed to stand at room temperature for 1 hour.

For enzyme detection the plates were first washed using the automated plate washer with Phosphate/Tween. Then, 150 µl of an ABTS solution was added to each well in the plate, and the rate of formation of the colored product read at 415 nm in a Bio-Rad Benchmark Microplate Reader. Readings took place every 15 seconds for 5 minutes. The ABTS solution was made by adding 17 mg of ABTS to 100 ml of 50 mM pH 5 citrate buffer. Immediately before use, 100 µl of 3% hydrogen peroxide was added to the solution to catalyze the enzymatic reaction.

The raw data for these experiments is presented in Table IV and Table V. Using the average of the normalized tests, a plot comparing the streptavidin plate to the silver plate was prepared. The data points shown on the graph in Figs. 7 are the average values of the normalized tests shown in Tables IV and V. The exception to this are the data points taken at Dilution 1, which were replaced by the values representing the asymptotic approach of the immunoassays as determined from the preceding points. From this, the maximum kinetic rate achievable in the silver plate is 375, versus 150 in the streptavidin plate. Due to the approach to a final value at high levels of enzyme, this difference is due to the amount of capture antibody on the plate. The silver plate binds more functional capture antibody, and thus is able to bind more enzyme when excess enzyme is present. Assuming that each antibody captures an average of 1.5 enzyme molecules, it is possible to estimate that the silver plate has approximately twice as many biotinylated antibody binding sites available. The ultimate detection limits of both plates is the same, however, and approaches femtomolar levels. This is the limit of the enzyme/substrate detection system being used.

TABLE IV

Silver Plate Immunoassay Data

Dilution Number	HRPO (mol/well)	Test 1		Test 2		Test 3		Test 1 Normalized	Test 2 Normalized	Test 3 Normalized	Average
		Antibody	Control	Antibody	Control	Antibody	Control				
1	2.73E-11	2.83E+02	2.52E+01	2.38E+02	1.83E+01	2.90E+02	2.55E+01	2.58E+02	2.20E+02	2.65E+02	2.47E+02
2	1.37E-11	3.87E+02	1.58E+01	3.46E+02	1.15E+01	3.94E+02	1.73E+01	3.71E+02	3.34E+02	3.76E+02	3.61E+02
4	6.83E-12	3.67E+02	9.82E+00	3.76E+02	7.55E+00	3.81E+02	1.22E+01	3.58E+02	3.68E+02	3.69E+02	3.65E+02
8	3.41E-12	3.36E+02	6.04E+00	3.44E+02	4.20E+00	3.63E+02	8.71E+00	3.30E+02	3.40E+02	3.54E+02	3.41E+02
16	1.71E-12	3.07E+02	5.74E+00	2.99E+02	3.37E+00	3.43E+02	7.77E+00	3.01E+02	2.96E+02	3.36E+02	3.11E+02
32	8.53E-13	2.67E+02	1.50E+01	2.70E+02	1.77E+00	2.90E+02	7.32E+00	2.52E+02	2.68E+02	2.83E+02	2.68E+02
64	4.27E-13	2.18E+02	2.83E+00	2.16E+02	2.11E+00	2.28E+02	5.53E+00	2.15E+02	2.14E+02	2.22E+02	2.17E+02
128	2.13E-13	1.61E+02	1.49E+00	1.53E+02	2.30E+00	1.50E+02	7.92E+00	1.60+02	1.51E+02	1.42E+02	1.51E+02
256	1.07E-13	7.38E+01	1.04E+00	7.18E+01	4.99E+00	7.39E+01	2.02E+00	7.27E+01	6.68E+01	7.18E+01	7.05E+01
512	5.33E-14	5.20E+01	1.35E+00	4.73E+01	2.57E+00	5.37E+01	2.32E+00	5.06E+01	4.48E+01	5.14E+01	4.89E+01
1024	2.67E-14	2.81E+01	1.49E+00	2.33E+01	4.83E+00	2.75E+01	3.26E+00	2.67E+01	1.85E+01	2.43E+01	2.31+01
2048	1.33E-14	1.20E+01	1.96E+00	1.34E+01	5.77E+00	1.43E+01	4.13E+00	1.00E+01	7.68E+00	1.02E+01	9.30E+00
4096	6.67E-15	6.66E+00	1.91E+00	1.05E+01	8.35E+00	8.61E+00	5.30E+00	4.75E+00	2.15E+00	3.31E+00	3.41E+00
8192	3.33E-15	4.73E+00	1.49E+00	1.03E+01	8.84E+00	3.03E+00	4.65E+00	3.24E+00	1.43E+00	-1.62E+00	1.02E+00
16384	1.67E-15	2.93E+00	2.41E+00	7.07E+00	6.65E+00	6.78E+00	6.25E+00	5.22E-01	4.28E-01	5.25E-01	4.92E-01
32768	8.33E-16	2.12E+00	3.86E+00	5.24E+00	5.19E+00	6.95E+00	8.48E+00	-1.74E+00	4.82E-02	-1.53E+00	-1.07E+00

TABLE V

Streptavidin Plate Immunoassay Data

Dilution Number	HRPO (mol/well)	Test 1		Test 2		Test 3		Test 1	Test 2	Test 3	Average
		Antibody	Control	Antibody	Control	Antibody	Control	Normalized	Normalized	Normalized	
1	2.73E-11	1.36E+02	6.50E+00	1.33E+02	8.55E+00	1.29E+02	5.50E+00	1.29E+02	1.24E+02	1.24E+02	1.26E+02
2	1.37E-11	1.74E+02	8.61E+00	1.24E+02	3.46E+00	1.75E+02	7.26E+00	1.65E+02	1.20E+02	1.68E+02	1.51E+02
4	6.83E-12	1.74E+02	1.96E+00	1.51E+02	2.29E+00	1.49E+02	2.06E+01	1.72E+02	1.49E+02	1.28E+02	1.50E+02
8	3.41E-12	1.62E+02	1.47E-01	1.34E+02	1.02E+00	1.38E+02	4.83E-01	1.62E+02	1.33E+02	1.38E+02	1.44E+02
16	1.71E-12	1.62E+02	-2.36E-01	1.40E+02	3.03E-02	1.30E+02	-2.53E-01	1.62E+02	1.40E+02	1.30E+02	1.44E+02
32	8.53E-13	1.49E+02	-8.89E-01	1.16E+02	6.89E-01	1.31E+02	-1.41E+00	1.50E+02	1.16E+02	1.33E+02	1.33E+02
64	4.27E-13	1.23E+02	-6.99E-01	1.28E+02	3.23E-01	1.05E+02	-1.26E+00	1.24E+02	1.28E+02	1.06E+02	1.19E+02
128	2.13E-13	1.04E+02	-1.47E-01	1.01E+02	-8.88E-01	8.99E+01	-2.05E+00	1.04E+02	1.02E+02	9.20E+01	9.93E+01
256	1.07E-13	3.86E+01	8.42E-01	5.01E+01	1.06E+00	4.32E+01	5.47E-01	3.78E+01	4.91E+01	4.26E+01	4.32E+01
512	5.33E-14	3.95E+01	1.37E+00	3.31E+01	7.59E-02	4.04E+01	6.97E-02	3.81E+01	3.31E+01	4.03E+01	3.72E+01
1024	2.67E-14	2.45E+01	1.38E+00	2.02E+01	-1.67E-01	2.45E+01	5.45E-01	2.31E+01	2.03E+01	2.40E+01	2.25E+01
2048	1.33E-14	1.29E+01	6.25E-01	9.56E+00	-8.19E-01	1.22E+01	1.06E-01	1.23E+01	1.04E+01	1.21E+01	1.16E+01
4096	6.67E-15	5.97E+00	-3.87E-01	4.76E+00	-1.42E+00	6.07E+00	-3.69E-01	6.36E+00	6.18E+00	6.44E+00	6.33E+00
8192	3.33E-15	2.75E+00	-3.01E-01	1.47E+00	-2.08E+00	3.18E+00	-6.08E-02	3.05E+00	3.55E+00	3.24E+00	3.28E+00
16384	1.67E-15	1.25E+00	-9.64E-01	1.64E-01	-1.36E+00	-2.02E+00	-9.85E-01	2.21E+00	1.52E+00	-1.04E+00	8.97E-01
32768	8.33E-16	1.07E+00	-1.22E+00	-2.82E-01	-1.87E+00	-9.76E-01	-2.00E+00	2.29E+00	1.58E+00	1.02E+00	1.63E+00

Claims

1. A bioassay plate having silver ions immobilized thereon.

2. The bioassay plate of Claim 1 wherein said plate is a polystyrene plate.

3. The bioassay plate of Claim 2 wherein said plate is a multi-well plate.

4. The bioassay plate of Claim 2 wherein said plate is a 96-well microplate.

5. A multi-well bioassay plate having silver ions immobilized thereon made by a method comprising:

a) functionalizing a multi-well bioassay plate to provide an amine - containing bioassay plate;

b) adding a polymerized glutaraldehyde to the wells of said plate and maintaining for a time and under conditions to provide a glutaraldehyde - activated bioassay plate;

c) rinsing said plate with an aqueous solution;

d) adding thiourea to the wells of said plate and maintaining for a time and under conditions whereby the thiourea reacts with the glutaraldehyde moiety of said glutaraldehyde-activated bioassay plate;

e) rinsing said plate with an aqueous solution; and

f) contacting said plate with silver ions for a time sufficient to immobilize said silver ions on said plate.

6. The multi-well bioassay plate of Claim 5 wherein said polymerized glutaraldehyde is prepared by allowing 25 wt % glutaraldehyde to polymerize at about 70°C for about 24 hours.

7. The multi-well bioassay plate of Claim 5 wherein said polymerized glutaraldehyde is maintained in the wells of said plate at about 37°C for about 24 hours.

8. The multi-well bioassay plate of Claim 5 wherein said thiourea is maintained in the wells of said plate at about 37°C for about 24 hours.

9. The multi-well bioassay plate of Claim 5 wherein said silver ions are added to said plate in the form of silver nitrate.

10. A method of making a multi-well bioassay plate having silver ions immobilized thereon comprising the steps of:

a) functionalizing a multi-well bioassay plate to provide an amine-containing bioassay plate;

b) adding a polymerized glutaraldehyde to the wells of said plate and maintaining for a time and under conditions to provide a glutaraldehyde - activated bioassay plate;

c) rinsing said plate with an aqueous solution;

d) adding thiourea to the wells of said plate and maintaining for a time and under conditions whereby the thiourea reacts with the glutaraldehyde moiety of said glutaraldehyde activated bioassay plate;

e) rinsing said plate with an aqueous solution; and

f) contacting said plate with silver ions for a time sufficient to immobilize said silver ions on said plate.

11. The method of Claim 10 wherein said polymerized glutaraldehyde is prepared by allowing 25 wt % glutaraldehyde to polymerize at about 70°C for about 24 hours.

12. The method of Claim 10 wherein said polymerized glutaraldehyde is maintained in the wells of said plate at about 37°C for about 24 hours.

13. The method of Claim 10 wherein said thiourea is maintained in the wells of said plate at about 37°C for about 24 hours.

14. The method of Claim 10 wherein said silver ions are added to said plate in the form of silver nitrate.

15. A method for detecting an antigen comprising the steps of:

a) incubating a multi-well bioassay plate having silver ions immobilized thereon with a biotinylated antibody having specificity for said antigen to provide a bioassay plate having said antibody immobilized thereon;

b) incubating said plate with a solution containing said antigen;

c) washing said plate with an aqueous solution;

d) incubating said plate with a labeled antibody having specificity for said antigen;

e) washing said plate with an aqueous solution; and

f) detecting said label, wherein detection of said label is indicative of the presence of said antigen.

16. A method for detecting a first antibody comprising the steps of:

a) incubating a multi-well bioassay plate having silver ions immobilized thereon with a biotinylated antigen that is reactive with said first antibody to provide a bioassay plate having said antigen immobilized thereon;

b) incubating said plate with an aqueous solution containing said first antibody;

c) washing said plate with an aqueous solution;

d) incubating said plate with an aqueous solution containing a labeled second antibody that binds to said first antibody;

e) washing said plate with an aqueous solution; and

f) detecting said label, wherein detection of said label is indicative of the presence of said first antibody.

17. A kit for the detection of a first antibody comprising a first container containing a bioassay plate having silver ions immobilized thereon.

18. The kit of Claim 17 further comprising a second container containing a biotinylated antigen that is reactive with said first antibody.

19. The kit of Claim 18 further comprising a third container containing a labeled second antibody that binds to said first antibody.

20. A kit for the detection of an antigen comprising a first container containing a bioassay plate having silver ions immobilized thereon.

21. The kit of Claim 20 further comprising a second container containing a biotinylated antibody having specificity for said antigen.

22. The kit of Claim 21 further comprising a third container containing an antibody having specificity for said antigen.

23. An apparatus for activating microplates comprising:

a) a housing;

b) a reagent addition/withdrawal chamber disposed in said housing, said reagent addition/withdrawal chamber including reagent and wash storage containers in communication with a manifold, said manifold in communication with dispense lines disposed to deliver wash and reagent to a microplate, and further including aspirate lines in communication with the manifold, said manifold in communication with a waste container, said aspirate lines disposed to aspirate spent reagent from said microplate;

c) an incubation chamber disposed in said housing, said incubation chamber including a means for vertically delivering a non-reactive sealing plate to said microplate, and a means for heating and agitating said microplate.

d) a means for horizontally conveying a microplate into and out of said addition/withdrawal chamber and between said addition/withdrawal chamber and said incubation chamber.

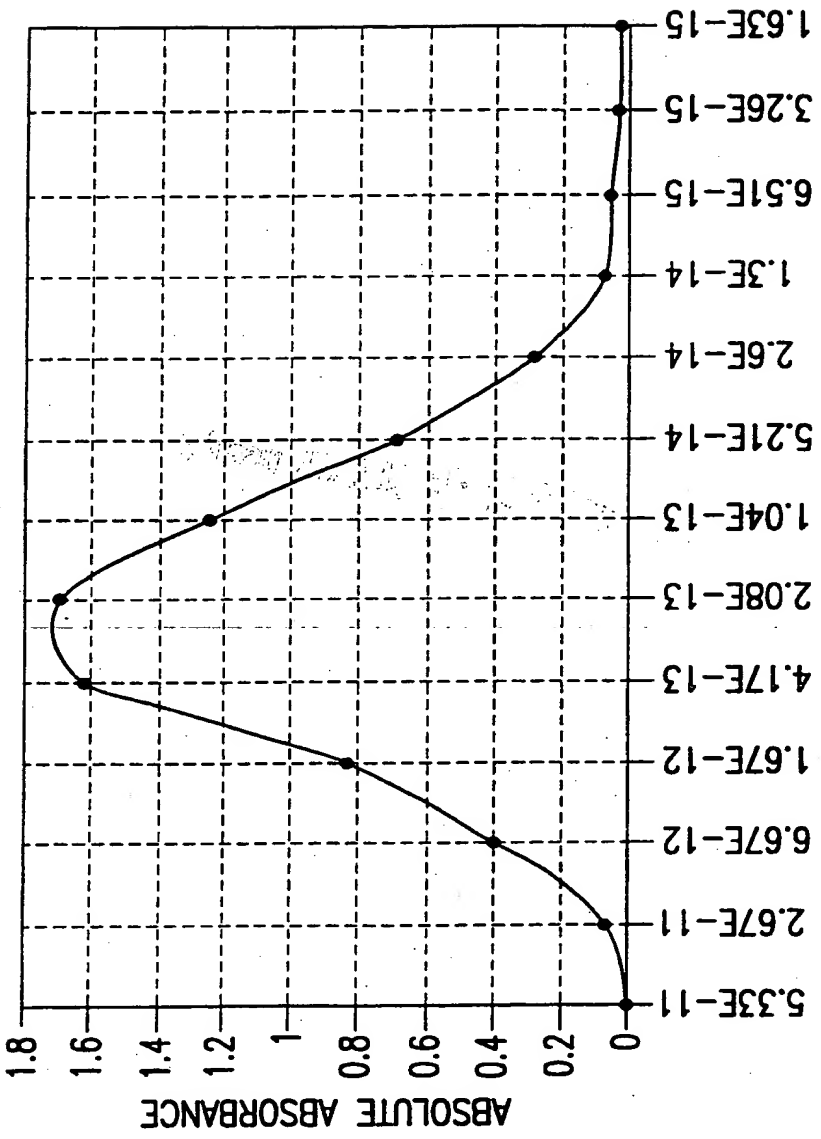
ABSOLUTE ABSORBANCE
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
MOLES BIOTINYLATED HRPO
1.63E-15
3.26E-15
6.51E-15
1.3E-14
2.6E-14
5.21E-14
1.04E-13
2.08E-13
4.17E-13
1.67E-12
6.67E-12
2.67E-11
5.33E-11

FIG.1

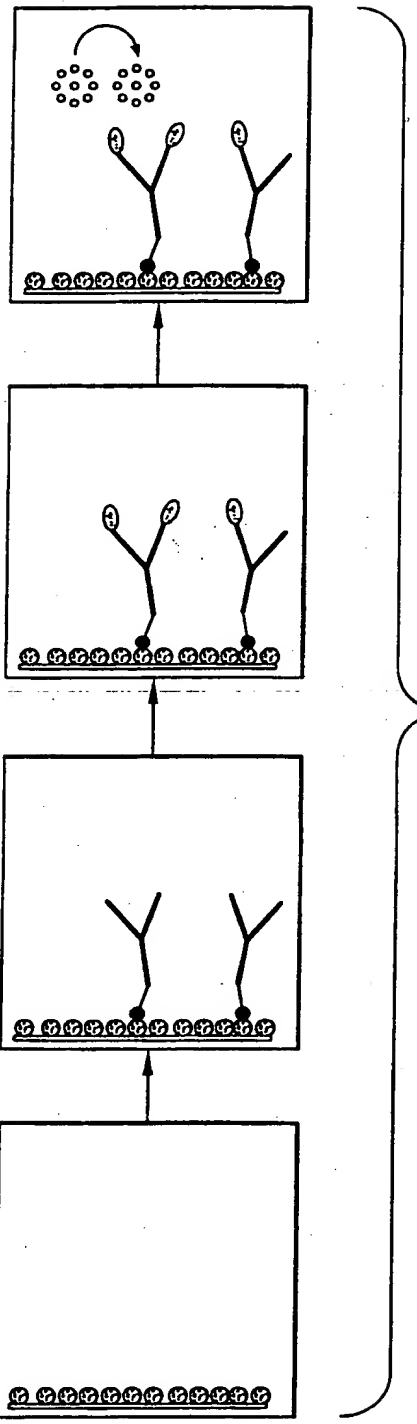

FIG.2

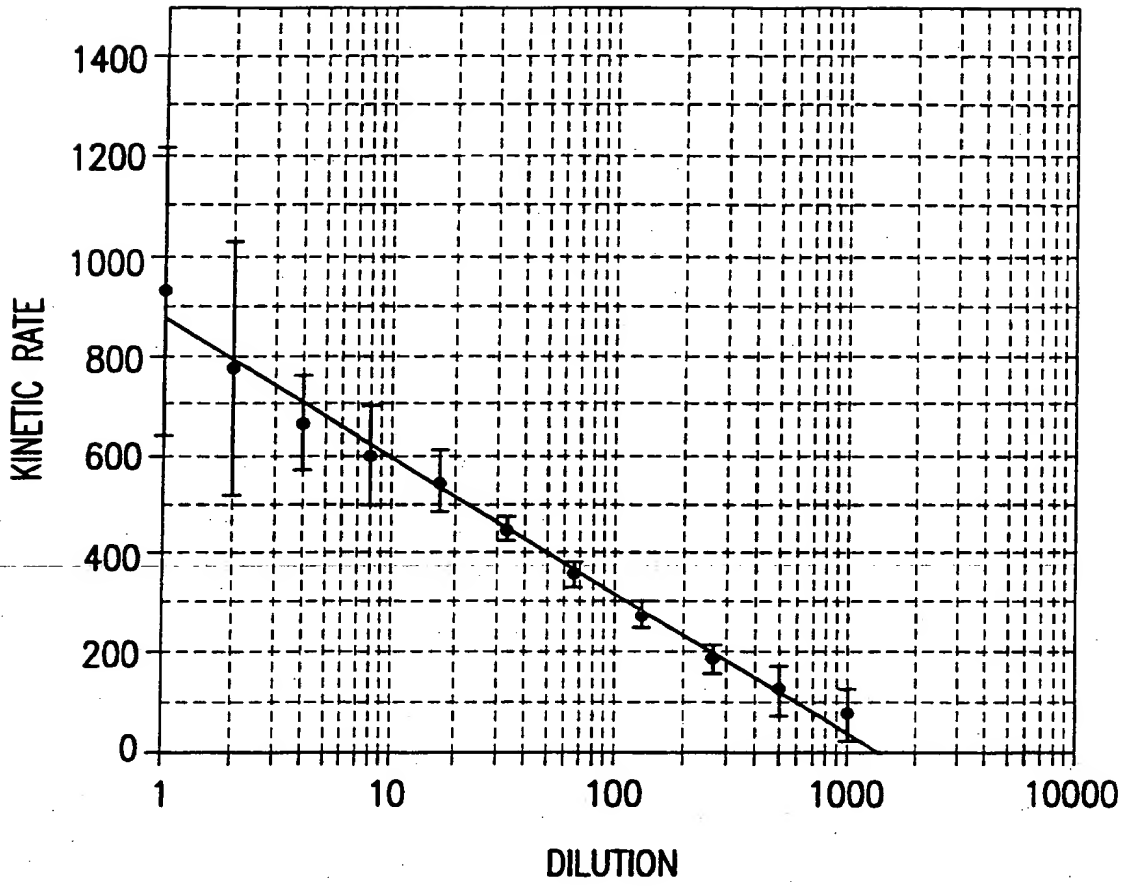
1400
1200
1000
800
600
400
200
0
KINETIC RATE
1
10
100
1000
10000
DILUTION

FIG.3

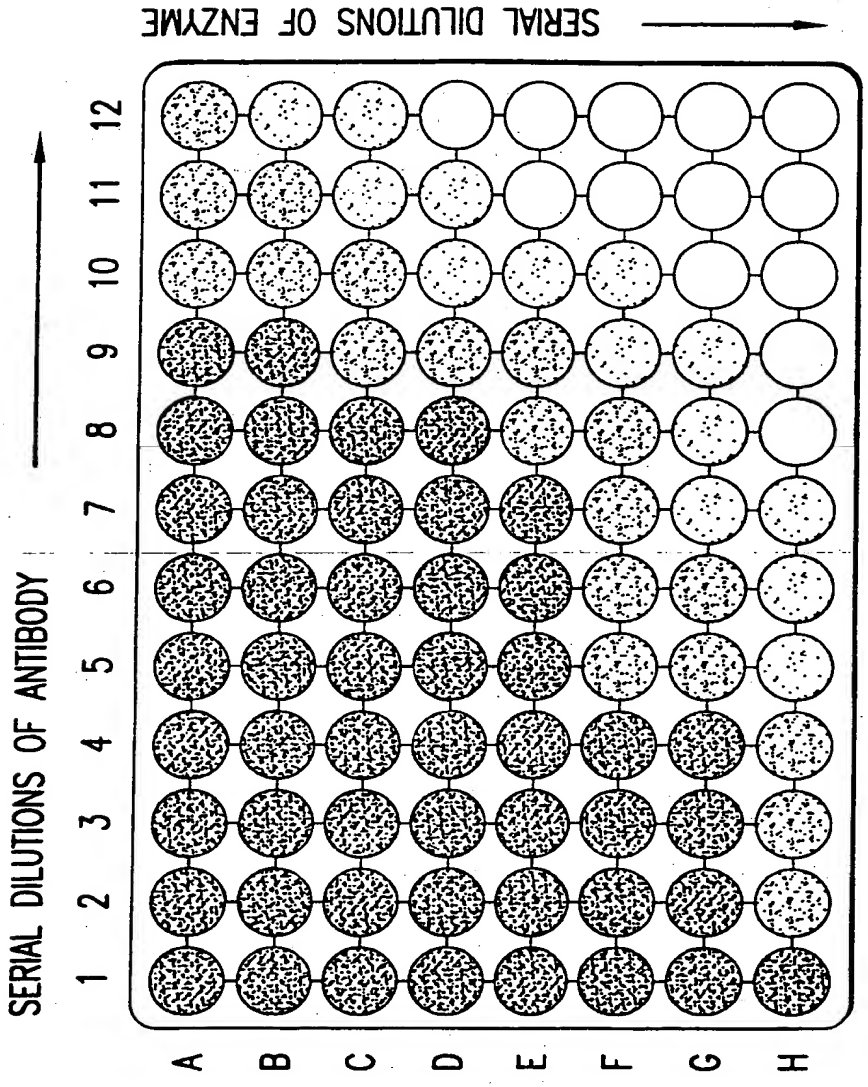
SERIAL DILUTIONS OF ENZYME
SERIAL DILUTIONS OF ANTIBODY
1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H

FIG.4

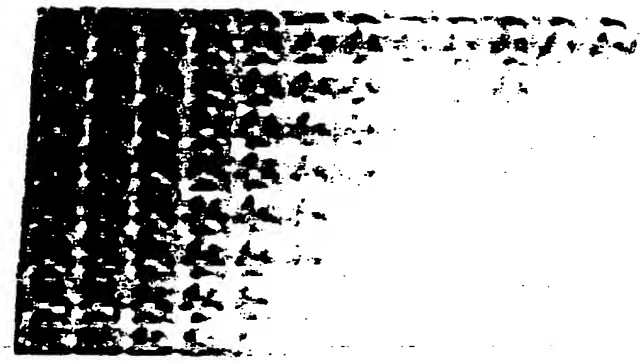

FIG.5

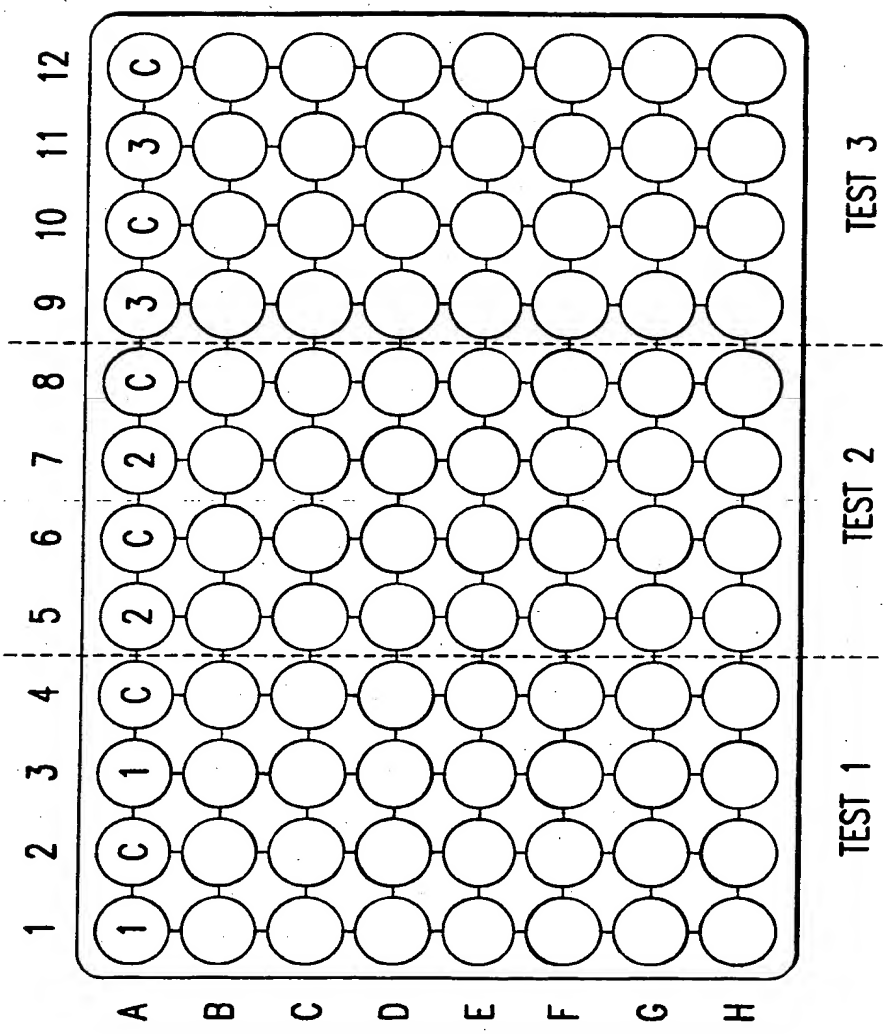

FIG.6

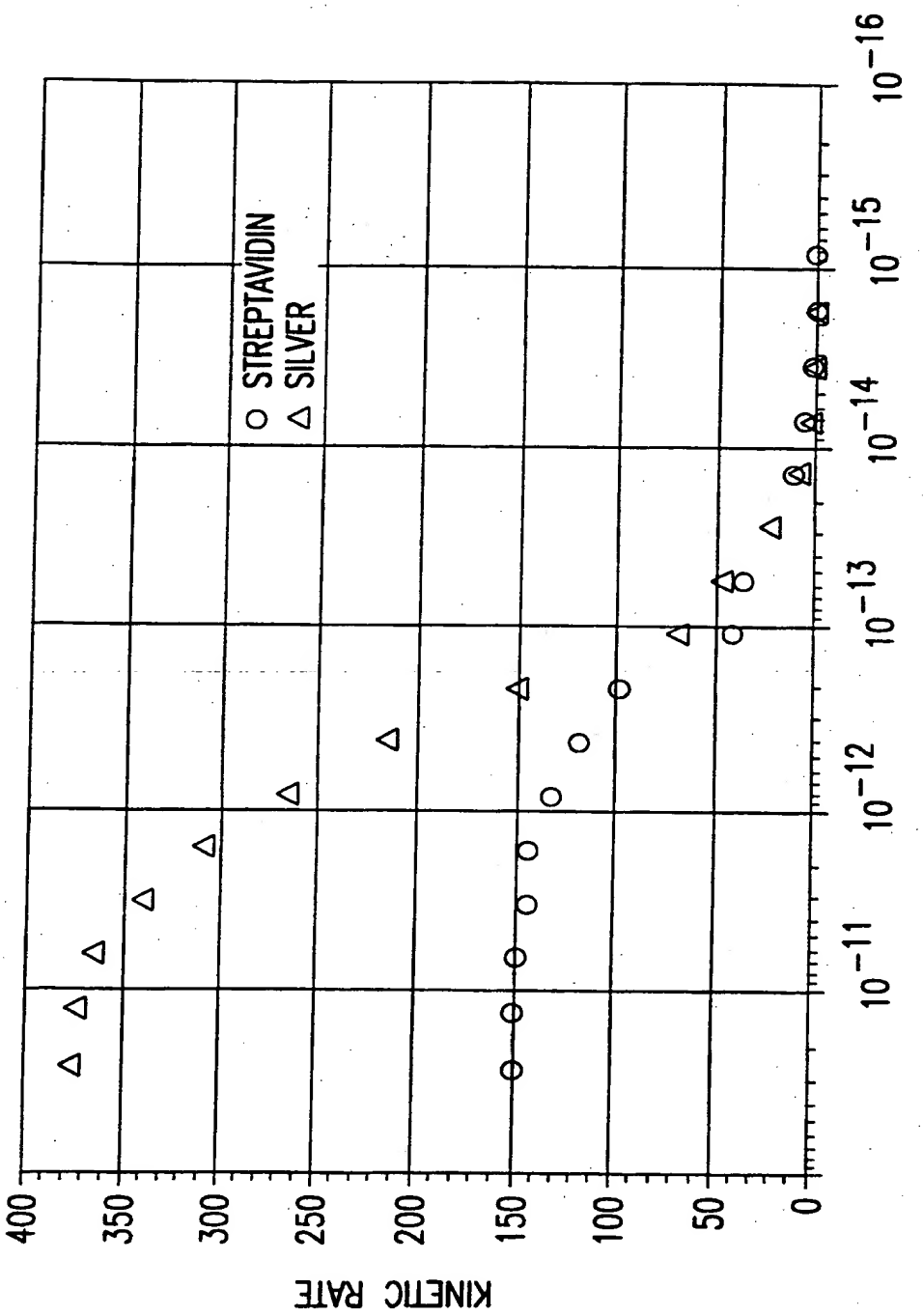
KINETIC RATE
400
350
300
250
200
150
100
50
0
STREPTAVIDIN
SILVER
10^{-11}
10^{-12}
10^{-13}
10^{-14}
10^{-15}
10^{-16}
MOLES ENZYME

FIG.7

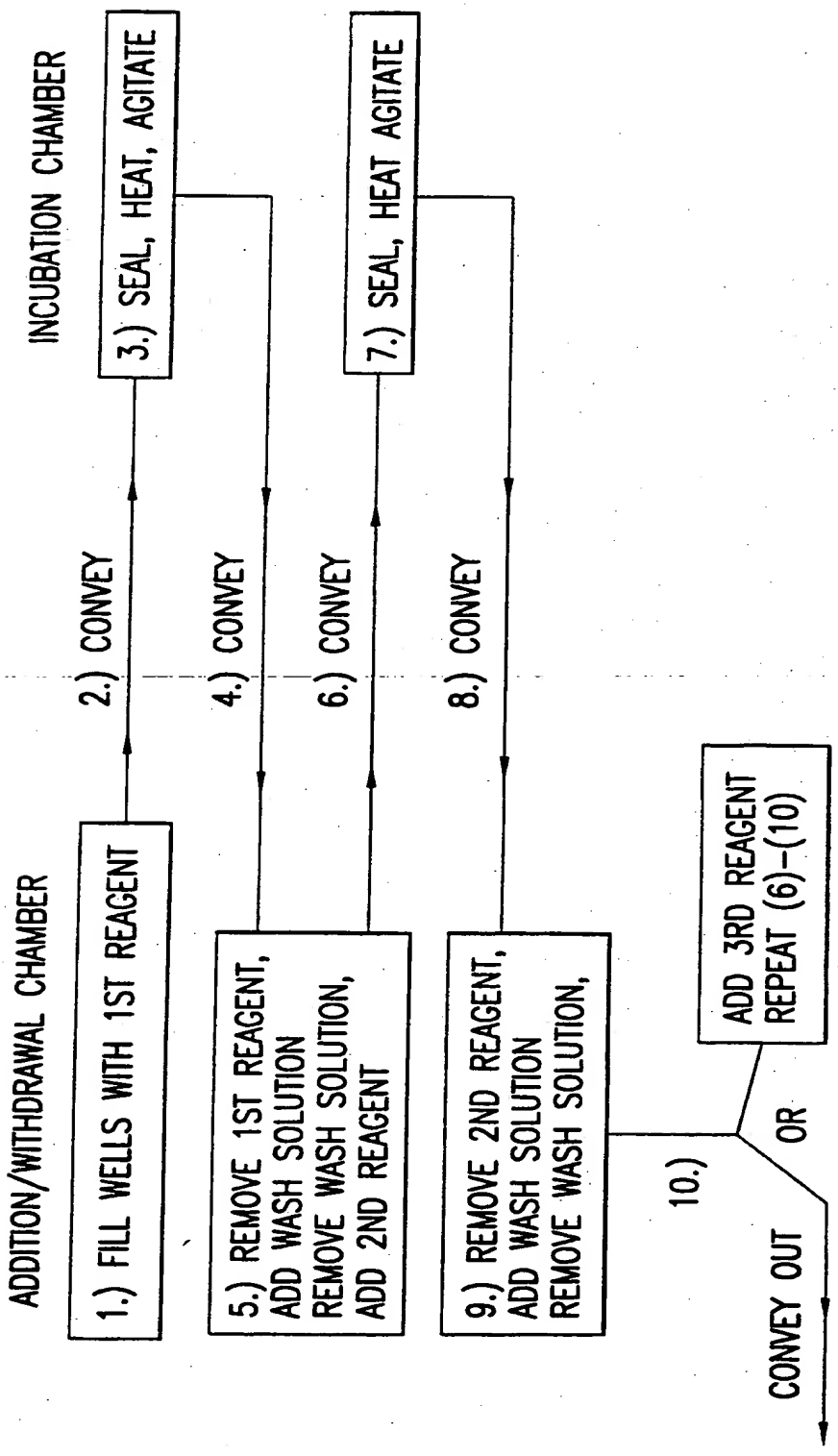
ADDITION/WITHDRAWAL CHAMBER
INCUBATION CHAMBER
1.) FILL WELLS WITH 1ST REAGENT
2.) CONVEY
3.) SEAL, HEAT, AGITATE
4.) CONVEY
5.) REMOVE 1ST REAGENT, ADD WASH SOLUTION REMOVE WASH SOLUTION, ADD 2ND REAGENT
6.) CONVEY
7.) SEAL, HEAT AGITATE
8.) CONVEY
9.) REMOVE 2ND REAGENT, ADD WASH SOLUTION REMOVE WASH SOLUTION,
10.)
ADD 3RD REAGENT REPEAT (6)-(10)
OR
CONVEY OUT

FIG.8

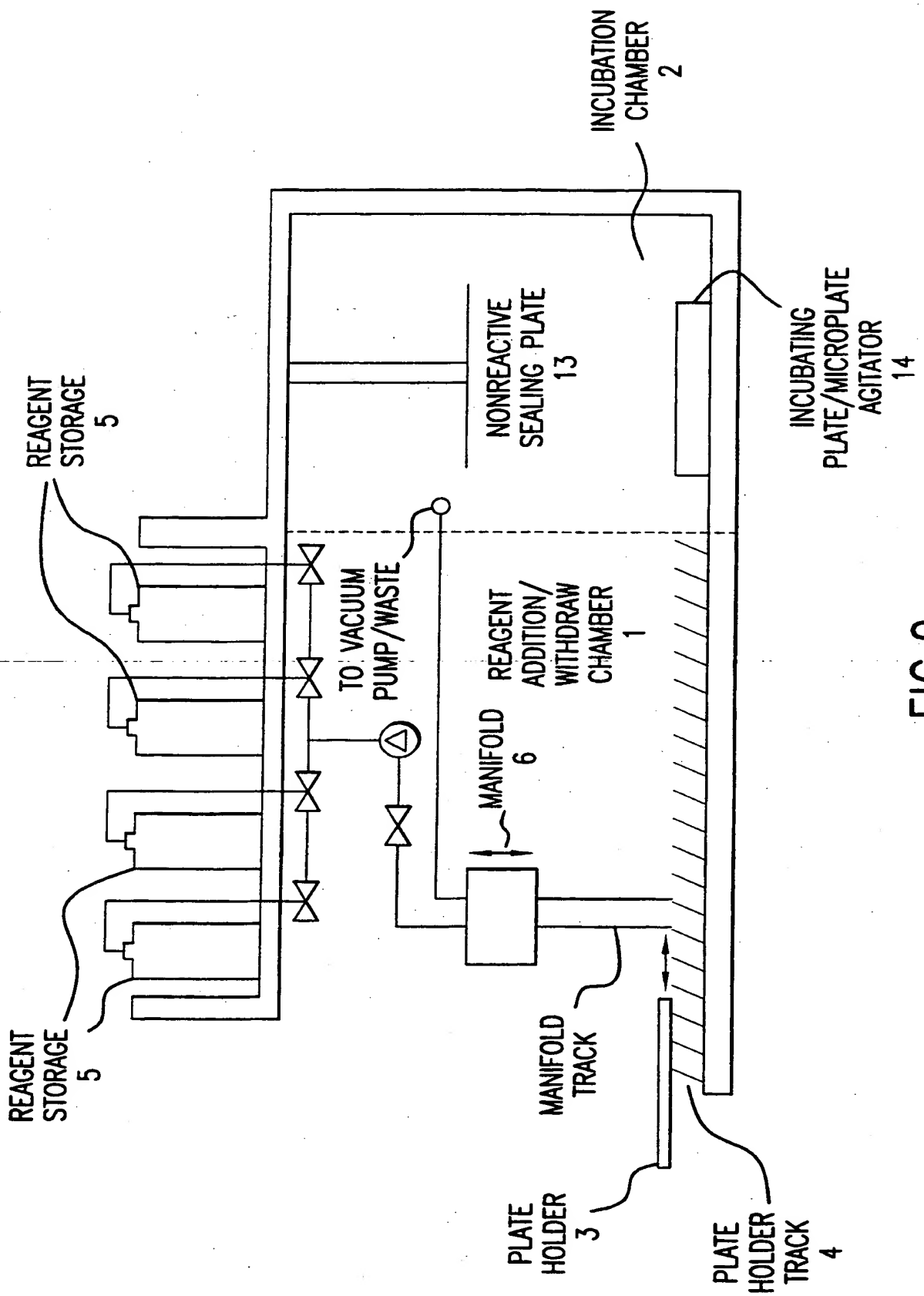
INCUBATION CHAMBER 2
NONREACTIVE SEALING PLATE 13
INCUBATING PLATE/MICROPLATE AGITATOR 14
REAGENT STORAGE 5
TO VACUUM PUMP/WASTE
REAGENT ADDITION/ WITHDRAW CHAMBER 1
MANIFOLD 6
REAGENT STORAGE 5
MANIFOLD TRACK
PLATE HOLDER 3
PLATE HOLDER TRACK 4

FIG.9

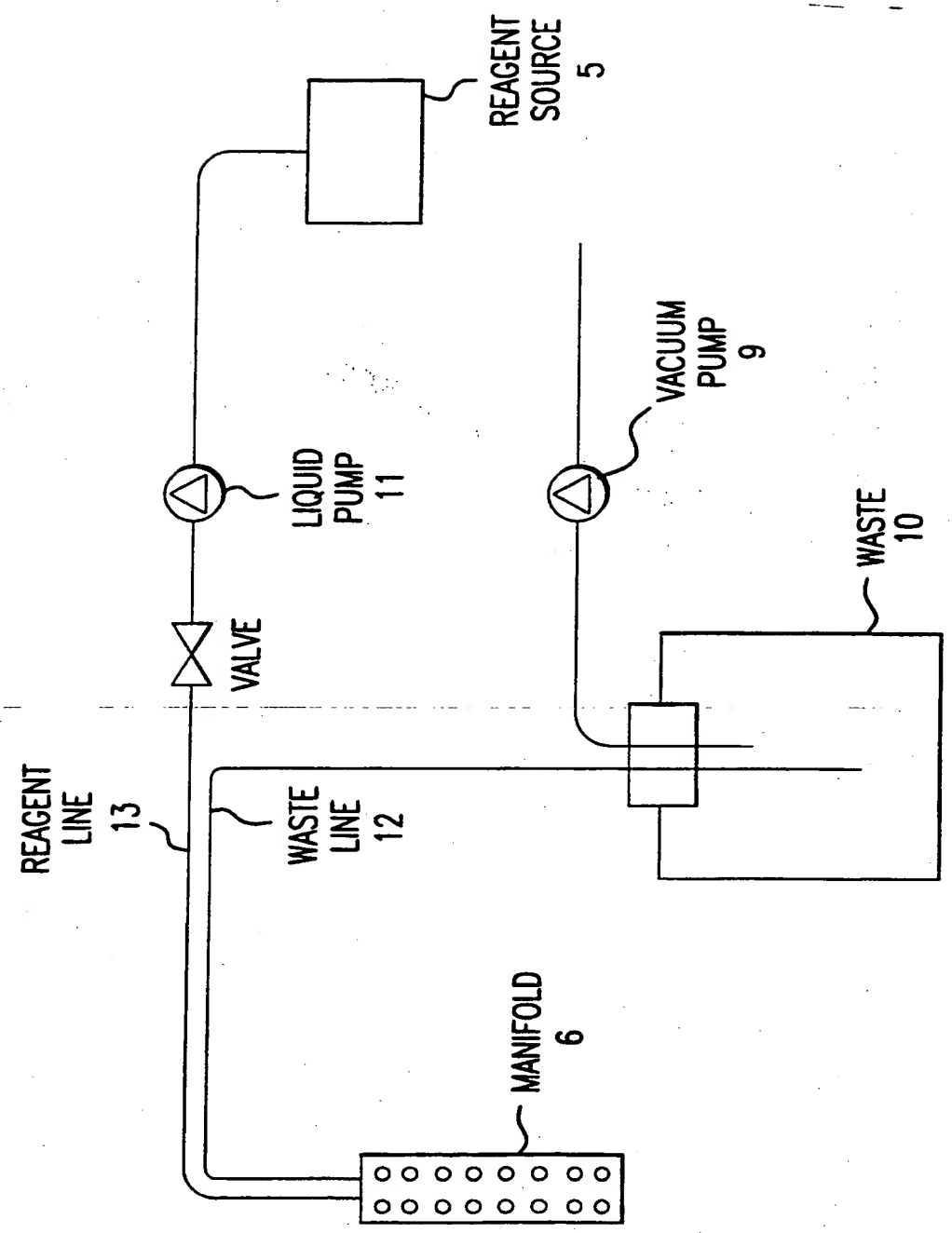
REAGENT SOURCE 5
VACUUM PUMP 9
LIQUID PUMP 11
WASTE 10
VALVE
REAGENT LINE 13
WASTE LINE 12
MANIFOLD 6

FIG.10

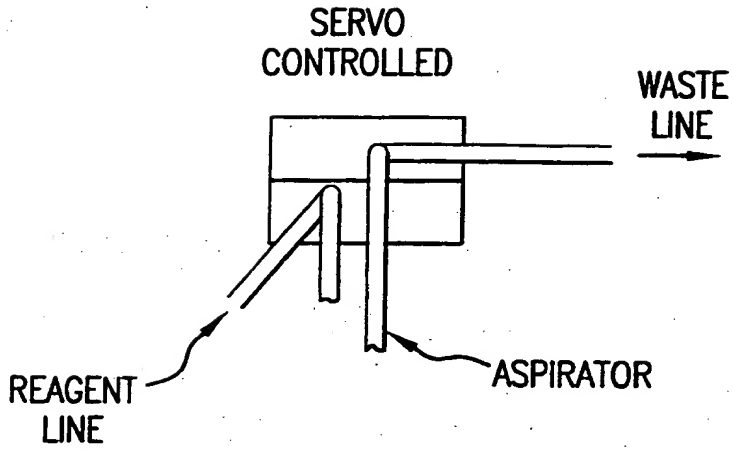

FIG.11A

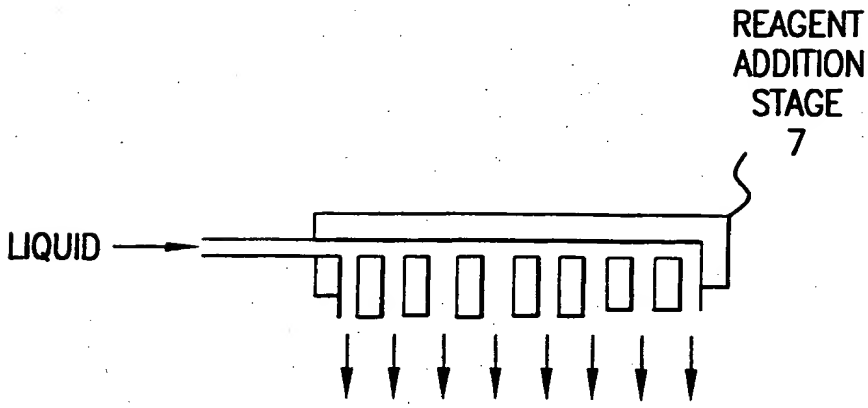

FIG.11B

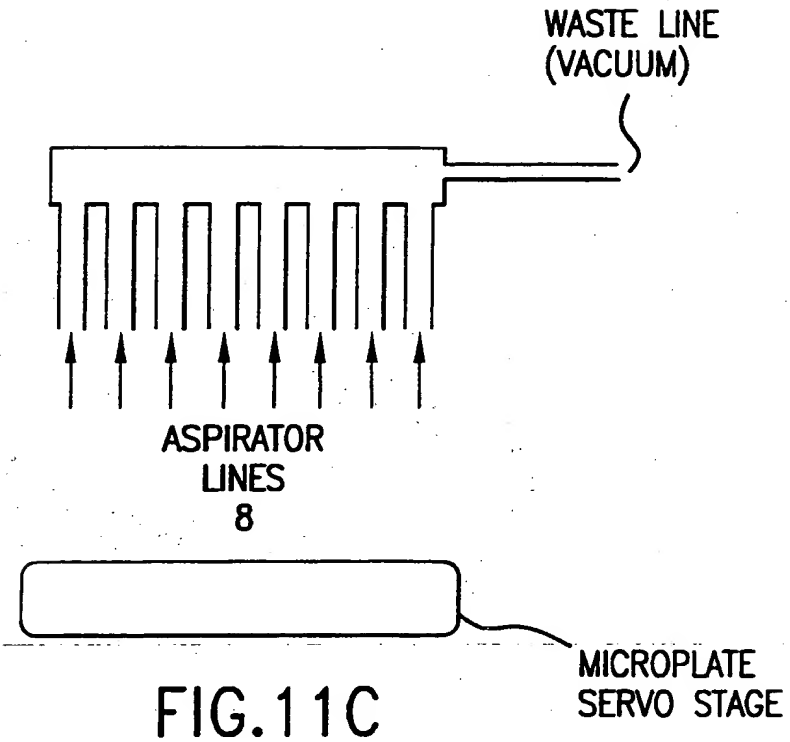
WASTE LINE
(VACUUM)
ASPIRATOR
LINES
8
MICROPLATE
SERVO STAGE

FIG.11C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23902

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :B01L 3/00; C12Q 1/68; B05D 5/12; G01N 33/53, 33/553, 33/549
US CL :422/99, 102; 427/123, 125; 435/6, 7.1; 436/525, 532
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/99, 102; 427/123, 125, 384, 389, 404, 414; 435/6, 7.1, 7.5, 7.6; 436/518, 524, 525, 531, 532, 808, 809

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,609,907 A (NATAN) 11 March 1997, col. 2, lines 60-67.	1 --- 2
Y	US 5,552,086 A (SIIMAN et al) 03 September 1996, col. 1, lines 49-50 and 64-65, col. 8, line 1, and col. 10, lines 64-67.	2, 5, 10
X --- Y	US 5,814,516 A (VO-DINH) 29 September 1998, columns 7 and 8.	1 --- 2, 5, 10
X --- Y	US 5,605,798 A (KOSTER) 25 February 1997, column 7, lines 54-65.	1 --- 3,4

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
21 JANUARY 2000	10 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer DWAYNE K. HANDY
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0661

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23902

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,705,813 A (APFFEL et al) 04 January1988, column 6, lines 4-20, column 7, lines 1-5, column 8, lines 22-31.	1-3, 15, 16
Y	US 4,775,636 A (MOEREMANS et al) 04 October 1988, column 3, lines 25-60, column 4, lines 7-50, column 6, lines 30-50.	1-3, 14-16
Y	US 5,543,332 A (LIHME et al) 06 August 1996, columns 2 and 3, also column 6.	15, 16
Y, P	US 5,908,746 A (SUZUKI et al) 01 JUNE 1999, column 1, lines 37-50, column 2, lines 29-31 and lines 56-57, column 5, lines 5-35, column 8, lines 19-45.	2-5, 10, 15, 16
Y, E	US 5,972,615 A (AN et al) 26 October 1999, column 7, lines 5-21, column 27, lines 48-52, column 28, lines 52-66, columns30-31.	2, 3, 15-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23902

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

EAST database
Search terms: microwell, microtiter, microfluidic, bioplate, bioassay, thiourea, coatings, surface derivitization, glutaraldehyde, silver, silver ions, silver nitrate, biotinylated antigen, biotinylated antibody

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 32011A-PCT	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US99/23902	International filing date *(day/month/year)* 14 OCTOBER 1999	Priority date *(day/month/year)* 14 OCTOBER 1998
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant ARIZONA BOARD OF REGENTS		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
31 MARCH 2000	14 FEBRUARY 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DWAYNE K. HANDY DEBORAH THOMAS PARALEGAL SPECIALIST Telephone No. (703) 308-0661

Form PCT/IPEA/409 (cover sheet) (July 1998)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/23902

I. Basis of the report

1. With regard to the elements of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-16 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 17-21 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawings:
pages 1-11 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/23902

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	2-16, 18-23	YES
	Claims	1, 17	NO
Inventive Step (IS)	Claims	5-14	YES
	Claims	1-4, 15-23	NO
Industrial Applicability (IA)	Claims	1-23	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claim 1 lacks novelty under PCT Article 33(2) as being anticipated by Natan. Natan teaches a bioassay plate with silver ions immobilized on it (Figure 1D, column 2, l. 60-65).

Claim 17 lacks novelty under PCT Article 33(2) as being anticipated by Moeremans et al. Moeremans teaches a kit for the detection of an antibody which has a first container having silver ions immobilized on it (column 6, lines 10-24 and column 3, lines 25-45 for the metal).

Claim 23 lacks an inventive step under PCT Article 33(3) as being obvious over Fanning et al. Fanning et al. teaches an automatic sample testing machine that contains: a housing (#20) with reagent addition and withdrawal station(s) (#300/400), an incubation station (#600), and means for transporting sample cards bewtween each of the stations. Fanning does not specifically recite each station (incubation, reagent addition/subtraction, etc.) inside a chamber, nor does Fanning teach the specific direction of delivery among the stations (horizontal, vertical, etc.). It would have been obvious to one of ordinary skill in the art, however, to place each station in a chamber. One would wish to avoid exposing the sample cards to heat (incubation) or pressure (fluid dispensing) while moving or running optical tests on the cards. As to the direction of delivery, it would be obvious to provide delivery in any direction as long as the cards are delivered and aligned properly with other components at each station.

Claims 15-22 lack an inventive step under PCT Article 33(3) as being obvious over Moeremans et al. in view of Apffel et al. Moeremans teaches a kit for antibody detection (see above) that contains a plate having silver ions immobilized on it. Moeremans does not teach the use of biotinylated antigen or antibody. Apffel shows a plate for MALDI analysis and teaches the use of biotinylated antibodies as binding partners in detection assays (column 6). It would have been obvious to one of ordinary skill in the art to provide biotinylated antigens/antibodies for use with the test kit of Moeremans. As to the selection of (Continued on Supplemental Sheet.)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): B01L 3/00; C12Q 1/68; B05D 5/12; G01N 33/53, 33/553, 33/549 and US Cl.: 422/99, 102; 427/123, 125; 435/6, 7.1; 436/525, 532

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
antigen/antibody pairs for the various containers (first, second, etc.), it would have been obvious to one of ordinary skill in the art to provide all the components necessary for a sandwich assay kit. Sandwich-type assays are common in the art and one of ordinary skill would only need to know the analyte of interest to choose which of the conjugate pair (antigen or antibody) to provide in the various containers.

Claims 2-4 lack an inventive step under PCT Article 33(3) as being obvious over Natan in view of Koster. Natan teaches the immobilization of silver ions on a surface for the purpose of MADLI analysis. Natan does not teach a microplate as the surface to be used. Koster teaches a process for DNA examination based on mass spectrometry on a surface which may include a solid support of metal or plastic, including microtiter plates (column 7, l. 54-64). It would have been obvious to one of ordinary skill in the art to use a microplate as taught by Koster as the surface taught by Natan. One would wish to use a microplate as the substrate since they provide a standard, well known surface with a plurality of sample locations for MALDI analysis.

Claims 5-14 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a method for immobilizing silver ions on a multiwell plate that includes a step which recites the use of thiourea.

Claims 1-23 meet the criteria set out in PCT Article 33(4), because the assay plate has use in biological assays.

---------- NEW CITATIONS ----------
US 5,762,873 A (FANNING et al) 09 JUNE 1998, see Abstract, columns 9-12.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23902

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :B01L 3/00; C12Q 1/68; B05D 5/12; G01N 33/53, 33/553, 33/549
US CL :422/99, 102; 427/123, 125; 435/6, 7.1; 436/525, 532
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/99, 102; 427/123, 125, 384, 389, 404, 414; 435/6, 7.1, 7.5, 7.6; 436/518, 524, 525, 531, 532, 808, 809

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,609,907 A (NATAN) 11 March 1997, col. 2, lines 60-67.	1 --- 2
Y	US 5,552,086 A (SIIMAN et al) 03 September 1996, col. 1, lines 49-50 and 64-65, col. 8, line 1, and col. 10, lines 64-67.	2, 5, 10
X --- Y	US 5,814,516 A (VO-DINH) 29 September 1998, columns 7 and 8.	1 --- 2, 5, 10
X --- Y	US 5,605,798 A (KOSTER) 25 February 1997, column 7, lines 54-65.	1 --- 3,4

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 JANUARY 2000	10 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DWAYNE K. HANDY Telephone No. (703) 308-0661

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23902

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,705,813 A (APFFEL et al) 04 January1988, column 6, lines 4-20, column 7, lines 1-5, column 8, lines 22-31.	1-3, 15, 16
Y	US 4,775,636 A (MOEREMANS et al) 04 October 1988, column 3, lines 25-60, column 4, lines 7-50, column 6, lines 30-50.	1-3, 14-16
Y	US 5,543,332 A (LIHME et al) 06 August 1996, columns 2 and 3, also column 6.	15, 16
Y, P	US 5,908,746 A (SUZUKI et al) 01 JUNE 1999, column 1, lines 37-50, column 2, lines 29-31 and lines 56-57, column 5, lines 5-35, column 8, lines 19-45.	2-5, 10, 15, 16
Y, E	US 5,972,615 A (AN et al) 26 October 1999, column 7, lines 5-21, column 27, lines 48-52, column 28, lines 52-66, columns30-31.	2, 3, 15-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23902

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

EAST database
Search terms: microwell, microtiter, microfluidic, bioplate, bioassay, thiourea, coatings, surface derivitization, glutaraldehyde, silver, silver ions, silver nitrate, biotinylated antigen, biotinylated antibody

PATENT COOPERATION TREATY

00 FEB 15 PM 2:28

From the INTERNATIONAL SEARCHING AUTHORITY

To: SORELL, LOUIS S.
MACLEOD, JANET M.
BAKER & BOTTS, LLP
30 ROCKEFELLER PLAZA
NEW YORK, NY 10112-0228

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Date of Mailing *(day/month/year)* 10 FEB 2000	
Applicant's or agent's file reference 32011A-PCT	**FOR FURTHER ACTION** **See paragraphs 1 and 4 below**
International application No. PCT/US99/23902	International filing date *(day/month/year)* 14 OCTOBER 1999
Applicant ARIZONA BOARD OF REGENTS	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Docketed
FOR 4/10/2000 By

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer for DWAYNE K. HANDY
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0661

Form PCT/ISA/220 (January 1994)★ *(See notes on accompanying sheet)*

1 Copy A Search Report & Reflects in Pocket ★

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 32011A-PCT	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US99/23902	International filing date *(day/month/year)* 14 OCTOBER 1999	(Earliest) Priority Date *(day/month/year)* 14 OCTOBER 1998
Applicant ARIZONA BOARD OF REGENTS		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (See Box I).

2. [ ] **Unity of invention is lacking** (See Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing

   [ ] filed with the international application.

   [ ] furnished by the applicant separately from the international application,

      [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.

   [ ] transcribed by this Authority.

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23902

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

EAST database
Search terms: microwell, microtiter, microfluidic, bioplate, bioassay, thiourea, coatings, surface derivitization, glutaraldehyde, silver, silver ions, silver nitrate, biotinylated antigen, biotinylated antibody

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23902

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	US 5,705,813 A (APFFEL et al) 04 January1988, column 6, lines 4-20, column 7, lines 1-5, column 8, lines 22-31.	1-3, 15, 16
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Y	US 5,543,332 A (LIHME et al) 06 August 1996, columns 2 and 3, also column 6.	15, 16
Y, P	US 5,908,746 A (SUZUKI et al) 01 JUNE 1999, column 1, lines 37-50, column 2, lines 29-31 and lines 56-57, column 5, lines 5-35, column 8, lines 19-45.	2-5, 10, 15, 16
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/23902

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :B01L 3/00; C12Q 1/68; B05D 5/12; G01N 33/53, 33/553, 33/549
US CL :422/99, 102; 427/123, 125; 435/6, 7.1; 436/525, 532
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/99, 102; 427/123, 125, 384, 389, 404, 414; 435/6, 7.1, 7.5, 7.6; 436/518, 524, 525, 531, 532, 808, 809

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	US 5,609,907 A (NATAN) 11 March 1997, col. 2, lines 60-67.	1 ----- 2
Y	US 5,552,086 A (SIIMAN et al) 03 September 1996, col. 1, lines 49-50 and 64-65, col. 8, line 1, and col. 10, lines 64-67.	2, 5, 10
X ----- Y	US 5,814,516 A (VO-DINH) 29 September 1998, columns 7 and 8.	1 ----- 2, 5, 10
X ----- Y	US 5,605,798 A (KOSTER) 25 February 1997, column 7, lines 54-65.	1 ----- 3,4

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
21 JANUARY 2000	10 FEB 2000

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	DWAYNE K. HANDY
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0661

Form PCT/ISA/210 (second sheet)(July 1992)*